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COALESCENCE AS A FACTOR IN SOLVENT STIMULATION OF PLASTID PHOSPHATIDASE C ACTIVITY[1]

Morris Kates and Paul R. Gorham

Abstract

Solvents which stimulate plastid phosphatidase C activity (e.g., ethyl ether, propyl ketone, and ethyl butyrate) have been observed (microscopically) to produce coalescence of lecithin and chloroplast phases, while those which do not stimulate the reaction (e.g., chloroform and petroleum ether) do not produce coalescence. In the ethyl ether-stimulated reaction, all of the original enzymatic activity was associated with the coalesced material, and the lecithin apparently formed an ether-insoluble complex with the chloroplasts; no enzymatic activity appeared in either the aqueous or ether phases. Ether extraction of chloroplasts alone did not liberate the enzyme from the plastids, and the presence of this stimulating solvent was still necessary for reaction with the substrate to occur. It is concluded that stimulating solvents achieve their effect primarily by causing substrate and plastid phases to coalesce and that the enzymatic reaction actually proceeds in the coalesced phase.

Introduction

Activation of a lecithin-hydrolyzing enzyme system by ethyl ether was first reported by Hanahan (1). He found that a lecithinase A–egg lecithin complex could be extracted into ether solution, and that subsequent hydrolysis of the substrate took place in the ether phase. Later, Hanahan, Rodbell, and Turner (3) showed that the enzyme remained attached to the ether-insoluble lecithin–lysolecithin precipitate which formed during the reaction Apparently, once the reaction was initiated, it proceeded in the insoluble phase of the system, the ether aiding in the reaction by removing the liberated fatty acid from the enzyme surface (3). An alcohol–ethyl ether medium was also found suitable for the operation of lecithinase A (3) as well as lecithinase D (5).

Stimulation of phosphatidase C[2] activity by ethyl ether has been observed in this laboratory (6, 7, 9). As will be shown in another paper (10), certain ketones and ethyl esters, and mixtures of methanol with petroleum ether or benzene are just as effective stimulators as ethyl ether, while chloroform, benzene, or petroleum ether are very poor stimulators.

[1]Manuscript received August 10, 1956.
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada.
Issued as N.R.C. No. 4156.

[2]The term "phosphatidase C" is used as defined previously (9).

In contrast to the initially homogeneous lecithinase A (3) and lecithinase D (5) systems, the plastid phosphatidase C system is heterogeneous, both plastids and substrate being insoluble in the suspending aqueous medium. Accessibility of substrate to the plastids is therefore a decisive factor affecting the rate of reaction. The visible coagulation of plastid and lecithin phases in ether-saturated systems (6) suggested that the reaction took place in the insoluble coagulum, and that ether promoted the adsorption of the substrate on the plastids (7). However, to substantiate this idea, it was necessary to determine whether all stimulating solvents produced coalescence while non-stimulating solvents did not, and whether enzymatic activity was still associated entirely with the solvent-treated plastids.

This paper presents evidence showing that coalescence of substrate and plastids occurs under the direct influence of stimulating solvents, and that the enzyme remains bound to the plastids during the reaction.

Materials and Methods

Plastid Preparations

Spinach, sugar beet, and cabbage chloroplast fragments were prepared by the high-speed centrifugation technique described previously (7). The preparations were probably contaminated by some nuclear and mitochondrial material (14).

Substrates

Egg lecithin was prepared by chromatography on alumina (4, 2). Commercial soybean phosphatide was partially purified as described previously (9). Analyses of these substrates are given in a previous publication (9). Synthetic (dioleyl)-L-α-lecithin (DOL) was the generous gift of Dr. Erich Baer (University of Toronto).

Solvents

All solvents used were reagent grade and, with the exception of ethyl ether, chloroform, and ethyl butyrate, were redistilled before use.

Analytical Methods

Phosphorus was determined by King's method (11), and choline (total and free) as described previously (7, 9).

Activity Measurement

Phosphatidase C (choline-liberating) activity of chloroplast fractions was measured as described previously (7, 8, 9).

Microscopy

Microscopic observations were made with a Zeiss bright field phase-contrast microscope, using a 40× objective and a 20× ocular. Photomicrographs were taken with a 35 mm. camera using Dupont microcopy film.

The following suspensions (in 0.1 *M* acetate buffer, pH 5.4) were used for microscopic observation:

(i) *Substrate alone.*—0.10% egg lecithin or 0.34% soybean phosphatide.

(ii) *Chloroplasts alone.*—0.36% (dry weight) cabbage chloroplasts.

(iii) *Substrate plus chloroplasts.*—Mixture containing 0.10% egg lecithin (or 0.34% soybean phosphatide) and 0.36% cabbage chloroplasts.

Each suspension was examined before and after shaking a 5.0 ml. portion with the following amounts of the various solvents: 0.75 ml. ethyl ether, 0.2 ml. *n*-propyl ketone, 0.2 ml. ethyl butyrate, 0.05 ml. chloroform, or 0.1 ml. petroleum ether (b.p. 35–55°). The amounts used were slightly in excess of saturation.

Blanks consisting of acetate buffer shaken with solvent were also examined to ascertain the appearance of the solvent droplets.

Experimental and Results

Microscopic Observations of the Effect of Solvents on Chloroplast–Lecithin Mixtures

When suspensions of egg lecithin alone, chloroplasts alone, or lecithin and chloroplasts together were allowed to stand, the suspended phases slowly settled to the bottom. Examination of these suspensions by phase-contrast microscopy revealed that the lecithin suspension (Fig. 1) contained clusters of irregularly-shaped lecithin particles; the chloroplast suspension (Fig. 2) contained small dark chloroplast fragments; and the lecithin–chloroplast mixture (Fig. 3) contained discrete lecithin and chloroplast phases, each having the same appearance as in separate suspension. No liberation of choline occurred with this latter mixture during 1 hour (7).

(*1*) *Effect of Ethyl Ether*

When the above mixtures were shaken with ethyl ether, in each case the suspended phases rapidly rose to the surface, leaving a clear aqueous phase below. Microscopic examination of the ether-treated suspensions showed that in the lecithin suspension (Fig. 4) the particles had a more regular shape (also note the myelin figure; the small dark particles are probably ether droplets); in the chloroplast suspension (Fig. 5), the fragments were coagulated into large amorphous masses; and the lecithin–chloroplast mixture (Fig. 6) no longer contained discrete lecithin and chloroplast phases but consisted of coagulated lecithin–chloroplast masses. Rapid liberation of choline occurred with ether-treated lecithin–chloroplast mixtures (7).

(*2*) *Effect of Propyl Ketone and Ethyl Butyrate*

When the lecithin–chloroplast mixture was shaken with propyl ketone or ethyl butyrate, the suspended phases likewise rose to the surface. Microscopic examination again showed the absence of discrete lecithin and

chloroplast phases and the presence of coagulated lecithin–chloroplast masses (Figs. 7 and 8, cf. Fig. 6). Rapid liberation of choline occurred in the presence of these solvents (10).

(3) *Effect of Chloroform and Petroleum Ether*

When the lecithin–chloroplast mixture was shaken with chloroform, discrete lecithin and chloroplast phases were still visible (Fig. 11). The lecithin phase appeared much the same as in a chloroform-treated lecithin emulsion alone (Fig. 10), while the chloroplast phase consisted of coagulated masses similar to those shown in Fig. 5. The petroleum-ether-treated lecithin–chloroplast mixture also exhibited discrete lecithin and chloroplast phases (Fig. 12, note large pool of excess solvent); the lecithin phase appeared very similar to that in the untreated lecithin–chloroplast mixture (Fig. 3).

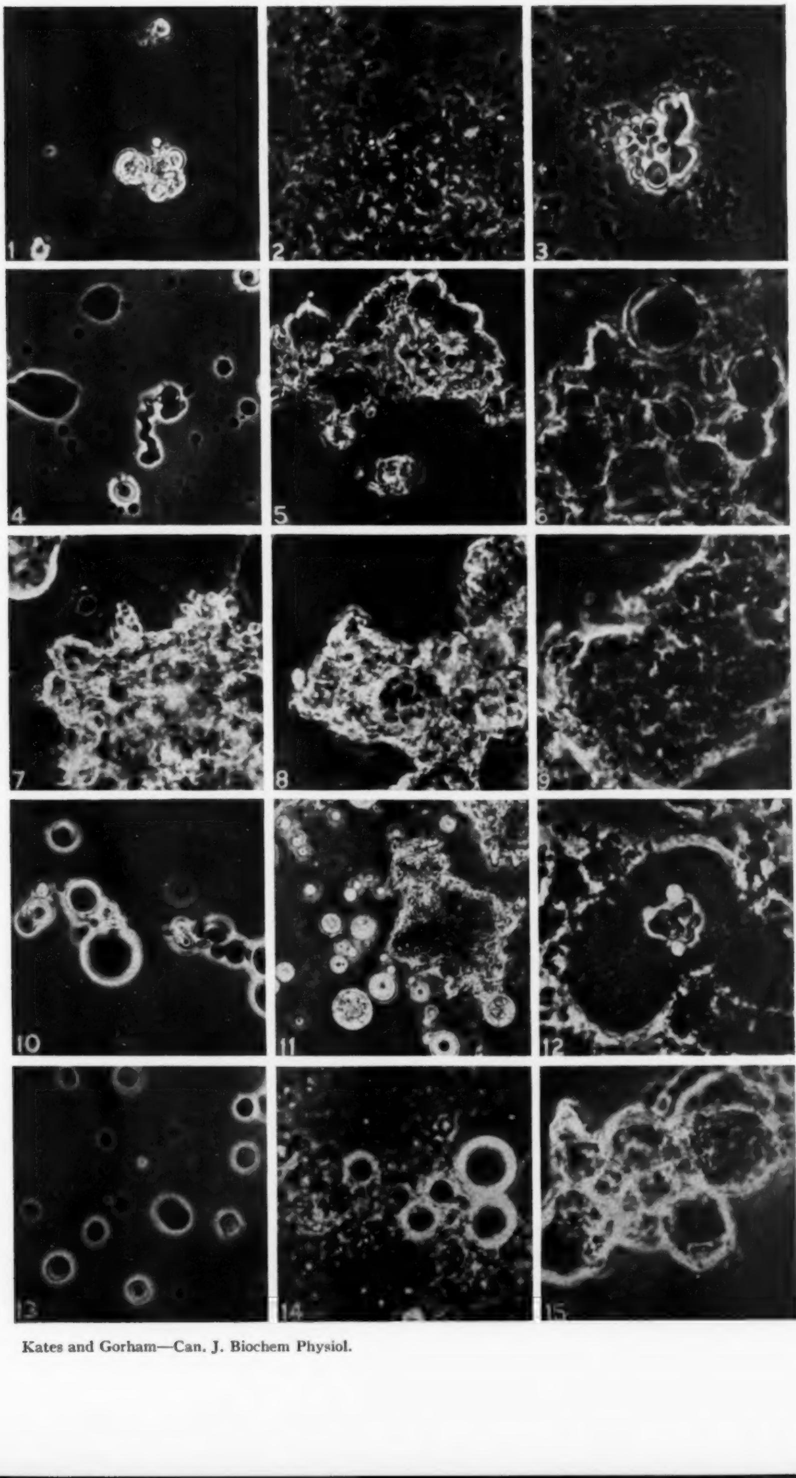
No liberation of choline occurred in the presence of these solvents and this was not due to inactivation of the enzyme (10). However, in the presence of 30% methanol, petroleum ether has been found to stimulate the enzymatic reaction to the same extent as ethyl ether (10). Microscopic examination of a lecithin–chloroplast mixture containing 30% methanol, after treatment with petroleum ether, showed the presence of coagulated chloroplast–lecithin masses and the absence of a discrete lecithin phase (Fig. 9), as was observed with all the stimulating solvents.

(4) *Soybean Phosphatide as Substrate*

The soybean phosphatide–chloroplast system was examined because liberation of nitrogenous bases from this substrate was found to occur rapidly in the *absence* of stimulating solvent, although ethyl ether did stimulate the reaction further (9). This substrate presumably contains a natural stimulating substance. Under the phase-contrast microscope, a soybean phosphatide suspension (Fig. 13) appeared similar to a solvent-treated egg lecithin suspension (Fig. 4). Examination of a mixture of soybean phosphatide and cabbage chloroplasts showed that the substrate, while still appearing as a discrete phase, was surrounded by or attached to the chloroplast fragments (Fig. 14), in contrast with the situation obtaining in the egg lecithin–chloroplast mixture (Fig. 3). When the soybean phosphatide–chloroplast mixture was shaken with ethyl ether, discrete substrate and chloroplast phases were no longer visible (Fig. 15), and the mixture appeared identical with the ether-treated egg lecithin–chloroplast system (Fig. 6).

FIGS. 1–15. Photomicrographs of buffered suspensions of lecithin and cabbage chloroplast fragments, alone or in combination with various solvents, taken a few minutes after mixing. Phase contrast, 400×. Abbreviations as follows: L = egg lecithin, S = soybean phosphatide, P = chloroplasts, E = ethyl ether, EB = ethyl butyrate, PK = *n*-propyl ketone, PE = petroleum ether (35–55°), M = methanol (30%), C = chloroform.

Fig. 1.	L	Fig. 6.	L + P + E	Fig. 11.	L + P + C
Fig. 2.	P	Fig. 7.	L + P + EB	Fig. 12.	L + P + PE
Fig. 3.	L + P	Fig. 8.	L + P + PK	Fig. 13.	S
Fig. 4.	L + E	Fig. 9.	L + P + PE + M	Fig. 14.	S + P
Fig. 5.	P + E	Fig. 10.	L + C	Fig. 15.	S + P + E



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Does Ether Extract Phosphatidase C from the Chloroplasts?

The following experiments were carried out in order to answer the question whether or not the enzyme was still bound to the plastids during the solvent-stimulated reaction, and also whether stimulating solvents achieve their effect by removing interfering or inhibiting substances:

A buffered suspension of chloroplasts was shaken with excess ethyl ether and immediately centrifuged. The ether and aqueous phases were removed separately, leaving the plastid phase (which rose to the water–ether interface) as a hard pellet. These operations required 20 minutes, and each of the separated phases was then immediately incubated with substrate and ether (where required). A control in which the chloroplast suspension was treated with ether but without separating the phases was also carried out. It was found that all of the activity of the ether-treated chloroplasts remained with the plastid phase and none was extracted into the ether or aqueous phase (Table I). Furthermore, the presence of the original ether phase did not affect the activity of the plastid phase.

TABLE I

DISTRIBUTION OF PHOSPHATIDASE C ACTIVITY AFTER ETHYL ETHER TREATMENT IN ABSENCE OF SUBSTRATE

Mixture containing 80 mg. spinach chloroplasts, 0.5 ml. 1 *M* acetate buffer (pH 4.8), and water to 4.5 ml. was treated with 10 ml. ethyl ether (see text); incubation mixtures contained indicated phases, 0.5 ml. 1 *M* acetate buffer (as required), 0.5 ml. of 5% lecithin suspension, water to 5.0 ml., and 10 ml. of ether (where required). Values are corrected for blank without substrate

Phase	Activity, mg. choline/10 min.
Complete system (phases not separated)	0.92
Plastid	1.14*
Plastid + ether	1.17*
Aqueous	0
Ether	0

*Activity higher than control since original aqueous phase, which contains an inhibitor present in the cell sap – cytoplasm (see (7)), has been removed.

To determine whether ether-extracted chloroplasts showed any activity in the complete *absence* of ether, the pellet of plastids obtained after the above treatment of a chloroplast suspension with ether was rapidly freed from solvent *in vacuo*, resuspended in buffer, and incubated with substrate in the absence of ethyl ether. No release of choline from the substrate occurred, although activity was still present, as demonstrated by the appearance of free choline when the incubation mixture was shaken with ether.

To determine whether the presence of substrate during the ether treatment would affect the above results, the experiment was modified as follows: A buffered mixture of chloroplasts and lecithin was shaken with excess ether

TABLE II

DISTRIBUTION OF PHOSPHATIDASE C ACTIVITY AFTER ETHYL ETHER TREATMENT IN PRESENCE OF SUBSTRATE

Mixture containing 78 mg. sugar beet chloroplasts, 0.25 ml. of 5% lecithin suspension, 0.5 ml. 1 *M* acetate buffer (pH 4.8), and water to 5.0 ml. was treated with 5 ml. of ethyl ether (see text); incubation mixtures contained the indicated phase, 0.5 ml. 1 *M* acetate buffer, with or without an additional 0.25 ml. substrate, water to 5.0 ml., and 5 ml. ethyl ether. Values are uncorrected for blank without substrate

	Mg. choline liberated/30 min.		
Phase	With extra substrate	Control, without extra substrate	Increment
Complete system (phases not separated)	3.42	1.85	1.57
Plastid	1.95	0.31	1.64
Aqueous	1.58*	1.40*	0.18
Ether	0.04	0.01	0.03

* Free choline here arose from enzymatic reaction during the centrifugation step before phases were separated; variations probably due to incomplete separation of the aqueous phase.

and immediately centrifuged. The phases were separated and then incubated with additional substrate in the presence of ether (where required). The additional substrate was added 15 minutes after the beginning of the experiment and subsequent incubation was for 15 minutes. In order to correct for hydrolysis of substrate in the 15-minute period during the separation of the phases, a control was run in which no additional substrate was added to the separated phases. Controls, in which the phases were not separated, were also carried out. Again, it was found that the activity of the ether-treated chloroplasts remained with the plastid phase and none appeared in the aqueous or ether phase (Table II).

Chemical Evidence for Chloroplast–Lecithin Complex Formation

In a previous study (9), evidence was obtained that suggested the formation of an ether-insoluble complex between chloroplasts and lecithin during the early stages of the ether-stimulated enzymatic reaction. In this work, however, the addition of acid to stop the enzymatic reaction might have affected the results obtained. An experiment was therefore designed so that the unacidified system could be analyzed as soon as possible after the beginning of the reaction.

A buffered mixture of cabbage chloroplasts and egg lecithin or (dioleyl)-lecithin was shaken with 10 ml. of ethyl ether and immediately centrifuged for 1 minute. The ether phase was removed by syringe pipette, the mixture was extracted twice more with 10-ml. portions of ether, and the ether phases were combined. The aqueous phase was then decanted from the pellet of ether-extracted chloroplasts and the latter was immediately extracted (6 minutes (egg lecithin), or 8 minutes (DOL) after beginning of experiment) with 10 ml. of ethanol–chloroform (4:1) at 70° for 5 minutes. The mixture

TABLE III

COMPLEX FORMATION BETWEEN LECITHIN AND CHLOROPLASTS

Mixtures (total volume, 5.0 ml.) of 113 mg. cabbage chloroplasts and 30.7 micromoles egg lecithin or 35.0 micromoles (dioleyl)-lecithin in 0.1 *M* acetate buffer (pH 5.5) were extracted with ethyl ether, and the separated phases were analyzed (see text)

	% of available P or choline					
	Egg lecithin*			(Dioleyl)-lecithin†		
Phase	Total P	Total choline	Choline/P molar ratio	Total P	Total choline	Choline/P molar ratio
Aqueous	1.2	27.2 (free)	—	5.6	31.2 (free)	—
Ether	74.0	39.8‡	0.54	44.0	20.8‡	0.48
Plastid (ethanol–chloroform extract)	21.8	13.2	0.61	50.4	31.0	0.62
Recoveries	97.0	80.2‡	—	100.0	83.0‡	—

*Reaction time, 6 minutes.
†Reaction time, 8 minutes.
‡Low choline values, probably due to interference by the large amounts of pigments in the ether extracts.

was centrifuged, the extract was decanted, and the residue was extracted again with 10-ml. and 5-ml. portions of solvent. The aqueous, ether and ethanol–chloroform solutions were then analyzed for phosphorus and choline, and the values were corrected for a blank determination with substrate omitted. A control determination with chloroplasts omitted showed that 99 to 100% of the substrate was extracted into the ether phase from aqueous suspension. In the presence of chloroplasts, however, a considerable amount of the available phosphorus and choline became associated with the plastids during ether extraction of the mixture (Table III). The low choline/P ratio (0.61–0.62) in the ethanol–chloroform extract of the plastids indicated the presence of some of the phosphatidic acid formed during the treatment with ether (8, 9). Analysis of the system immediately after addition of ether would have reduced the amounts of phosphatidic acid to very small values, but in practice this could not be achieved owing to the rapidity of the enzymatic reaction and the appreciable time necessary to separate the phases. Nevertheless, the fact that 13% and 31% of egg lecithin and DOL, respectively, were still associated with the plastids after repeated extraction with ether shows that complex formation between lecithin and plastids occurs under the influence of this solvent.

Discussion

The results of the microscopic observations show that all solvents which stimulate plastid phosphatidase activity cause substrate and plastids to coalesce (Figs. 6, 7, 8, and 9), while those that do not stimulate the reaction do not produce coalescence (Figs. 11 and 12). Furthermore, soybean

phosphatide, which probably contains a natural stimulating substance (9), shows a greater affinity towards the plastids (Fig. 14) than does purified egg lecithin (Fig. 3). Finally, the coalesced material contains all of the original enzymatic activity (Tables I and II), as well as lecithin in the form of a complex with the plastids (Table III). It may therefore be concluded that stimulating solvents achieve their effect primarily by causing substrate and plastid phases to coalesce, and that enzymatic reaction actually proceeds in the coalesced phase. The mechanism underlying the phenomenon of coalescence is discussed fully in another paper (10).

The present results have a bearing on the behavior of another chloroplast enzyme, chlorophyllase. This enzyme has been found to operate in moist ether (15) and in aqueous solutions of methanol, ethanol, or acetone (12, 13, 15). Furthermore, both this enzyme and its substrate, chlorophyll, are insoluble in water, and it has been recognized that "accessibility of substrate to enzyme plays an important part in determining the rate of reaction" (13). It would be interesting to reinvestigate the chlorophyllase system in the light of the present knowledge concerning solvent stimulation of chloroplast phosphatidase C.

Acknowledgment

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EFFECTS OF SOLVENTS AND SURFACE-ACTIVE AGENTS ON PLASTID PHOSPHATIDASE C ACTIVITY[1]

MORRIS KATES

Abstract

Large stimulations of plastid phosphatidase C activity were produced by (i) linear aliphatic ethers, ketones, and esters; (ii) mixtures of methanol with ethyl ether, petroleum ether, or benzene; and (iii) anionic detergents. Cyclic ethers, ketones, or esters and also alcohols, aldehydes, halogenated compounds, or hydrocarbons produced relatively small stimulations; cationic or non-ionic detergents produced little or no stimulation. The stimulations produced by linear aliphatic ethers, ketones, or esters varied greatly with the chain length of the hydrocarbon groups attached to the oxygen functional group: in each respective class, the most effective solvents were ethyl ether, *n*-propyl ketone, methyl pentyl ketone, ethyl butyrate, and butyl acetate. The most probable mechanism for explaining the stimulation effect is concluded to be one which involves adsorption of the stimulating solvents at the surfaces of the lecithin and plastid particles in a manner which makes these surfaces lipophilic and thus mutually attracting.

In general water-insoluble solvents (ethers, ketones, esters, hydrocarbons) produced relatively little inactivation of enzymatic activity, while water-miscible solvents (alcohols, cyclic ethers, etc.) were strongly inactivating, especially at high concentrations (60–70%). Anionic detergents were not inhibitory in the concentration range in which they were stimulating ($< 0.02\ M$), but were inhibitory at higher concentrations; cationic detergents were strongly inhibitory at all concentrations.

It is concluded that extraction of plant phosphatides without concomitant enzymatic degradation should be possible by the use of *i*-propanol or *n*-propanol as solvent.

Introduction

Previous studies in this laboratory (12, 13, 15) have shown that the enzymatic hydrolysis of glycerolphosphatides by plastid phosphatidase C[2] is greatly stimulated by ethyl ether; small stimulations were also observed with acetone and with 'Dreft', whereas *n*-butyl ether, dioxane, methanol, butanol, chloroform, or petroleum ether produced little or no activation (12). It was of interest to determine whether the stimulation effect is specific for ethyl ether, and, if not, what other solvents or surface-active agents can produce similar stimulations. Such information would be useful in deriving a mechanism to explain the stimulation effect. Furthermore, knowledge concerning both the activating and inactivating effects of solvents on plant phosphatidases was required to develop procedures for extracting plant phosphatides without accompanying enzymatic degradation (see (13)).

This report presents the results of a study of the effects of solvents and surface-active agents on the phosphatidase C activity of chloroplasts. A mechanism which explains the stimulation phenomenon is also presented.

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Issued as N.R.C. No. 4159.
[2]The term "phosphatidase C" is used as defined previously (15).

Materials and Methods

Plastid Preparations

Spinach or sugar beet chloroplast fragments were prepared by the high-speed centrifugation procedure described previously (13).

Substrate

The lecithin used in these studies was prepared from egg yolk by the method of Hanahan, Turner, and Jayko (9) or by the modified method of Hanahan (6). Analytical data were the same for the products of both procedures and were given in a previous paper (15). Although this substrate contains lysolecithin-like material ((15), see also (18)), it was used in the present study because it was relatively easy to prepare, and the presence of the impurity did not affect the pattern of hydrolysis (15).

Solvents

Most of the solvents used were reagent grade and were usually redistilled before use. Practical grade solvents were always redistilled. Dioxane was filtered repeatedly through alumina before use to remove peroxides. Diethyl ether (anhydrous reagent grade) was used without further purification; distillation of diethyl ether over sodium had no effect on its stimulating activity. Methyl ether (gaseous, Ohio Chemical and Manufacturing Company) was used directly from the cylinder.

Surface-active agents

The non-ionic detergents, Span 60 (sorbitan monostearate), Atlas G2152 (polyoxyethylene stearate, high molecular weight), and Myrj 45 (polyoxyethylene stearate, low molecular weight) were obtained from Atlas Powder Company. Sodium dodecyl sulphate (SDS) was obtained from Bios Laboratories Inc.; sodium desoxycholate (SDC) and sodium taurocholate from Difco Laboratories Inc.; stigmasterol and sitosterol from Nutritional Biochemicals Corp.; soybean sitosterols from Glidden Co.; and cetyl pyridinium chloride (CPC) from N.S. Merrel Co.

Activity Measurement

Lecithinase activity (choline-liberation) of chloroplast fractions was measured as described previously (13, 14, 15). In some of the present studies, the order of addition of the components in the assay system was found to influence the rate of reaction. The order of addition was standardized as follows:

Stimulation Studies

(*a*) *Solvent or detergent alone.*—Buffer, substrate, chloroplast suspension, followed by solvent; or buffer, detergent, substrate, followed by chloroplasts.

(*b*) *Combination of solvents or substances.*—Buffer, water-soluble solvent, or detergent, substrate, chloroplasts, followed by ethyl ether or other water-insoluble solvent.

Inactivation Studies

(*a*) *Water-soluble solvents.*—Solvent, chloroplast suspension, then after centrifugation and removal of supernatant, buffer, substrate and ethyl ether.

(*b*) *Water-insoluble solvents.*—Buffer, chloroplast suspension, low-stimulating solvent, followed by incubation with substrate and ethyl ether; with high-stimulating solvents (other than ethyl ether), ethyl ether was omitted during the incubation.

Blanks (substrate omitted) as well as ethyl ether controls were run in each activity determination; the results were corrected for the blanks and expressed as percentages of the ether control. Activities less than 10% of the ether control are considered to be of doubtful significance.

Variations in the phosphatidase C activity of spinach and sugar beet chloroplasts with the seasons were observed: in general, chloroplast preparations had higher activities in the winter (Jan.–Feb.) than in the summer or early fall. The present results, obtained with spinach and, to some extent, with sugar beet chloroplasts may not apply in detail to plastid preparations from other sources.

Experimental and Results

Stimulation of Chloroplast Phosphatidase C Activity

(*a*) *Individual Organic Solvents*

The stimulating ability of a variety of organic solvents, relative to that of ethyl ether, is given in Table I. Although the number of solvents investigated is by no means all-inclusive, it is possible to draw the following conclusions:

(1) In addition to ethyl ether, other aliphatic linear ethers, as well as aliphatic linear ketones and esters produce relatively large stimulations; alcohols, aldehydes, halogenated solvents, and hydrocarbons, however, produce relatively small stimulations.

(2) The stimulating ability of a solvent does not depend directly on its solubility in water (cf. butanol with ethyl ether), but all solvents with high stimulating ability have low solubilities in water.

(3) For each class of stimulating solvents the effect is dependent on the nature of the alkyl groups (R) attached to the oxygen functional group: (i) Cyclic ethers, ketones, or esters produce little or no effect, while the corresponding linear compounds produce large stimulations (cf. tetrahydrofuran, cyclopentanone, and butyrolactone with ethyl ether, diethyl ketone, and ethyl acetate, respectively). (ii) The stimulating effects of homologous linear, straight chain ethers, ketones, or ethyl esters vary markedly with the length of the alkyl substituents R, as shown in Fig. 1. With symmetrical ethers, maximum stimulation occurs when R is ethyl, with symmetrical ketones when R is propyl, with methyl ketones when R is pentyl, and with ethyl esters when R is propyl. It is interesting to note that the symmetrical ketone and the methyl ketone producing maximum stimulation have

TABLE I

STIMULATION OF PLASTID PHOSPHATIDASE C ACTIVITY BY VARIOUS SOLVENTS

Reaction mixtures (100–105 mg. spinach chloroplasts or 116–120 mg. sugar beet chloroplasts, 32–33 micromoles lecithin, and acetate buffer (0.1 *M*, pH 4.7) in 5.0 ml. total volume) were shaken with 1 ml. of each solvent and incubated 10 minutes at 25°

Solvent	Solubility (20°) g./100 ml. water	Relative activation, % ethyl ether control*	
		Spinach	Sugar beet
None		1	< 1
Ethers			
Methyl ether†	3700 (ml. gas at 18°)	6 ± 1	—
Ethyl ether‡	7.5	100§	100§
i-Propyl ether	0.2	53	—
n-Propyl ether	0.25 (25°)	33	—
n-Butyl ether	Slightly soluble	15	14
Tetrahydrofuran	∞	18	—
p-Dioxane	∞	1	10
Ketones			
Acetone	∞	14 ± 2	7 ± 1
Diethyl ketone	4.7	75 ± 2	85
Di-*n*-propyl ketone	Insoluble	104 ± 4	—
Di-*n*-butyl ketone	Very slightly soluble	41	—
Di-*i*-butyl ketone	Insoluble	36	—
Methyl ethyl ketone	35.3 (10°)	29 ± 1	27
Methyl-*i*-propyl ketone	Slightly soluble	47 ± 0	—
Methyl-*n*-propyl ketone	Slightly soluble	59 ± 1	—
Methyl-*i*-butyl ketone	1.9	87 ± 1	90
Methyl-*n*-pentyl ketone	Very slightly soluble	101	—
Methyl-*n*-hexyl ketone	Insoluble	91	—
Cyclopentanone	Slightly soluble	4	—
Cyclohexanone	2.4 (31°)	4	—
Esters			
Ethyl formate	11.8 (25°)	38 ± 3	—
Ethyl acetate	8.6	72 ± 1	78
Ethyl propionate	2.4	93 ± 2	100
Ethyl butyrate	0.68	96 ± 4	—
Ethyl hexanoate	0.0015	62	—
Ethyl octanoate	0.063 (25°)	25	—
Butyl acetate	0.5 (25°)	96	—
Butyrolactone	∞	5	—
Alcohols			
Methanol	∞	4 ± 1	10
Ethanol	∞	26	—
i-Propanol	∞	35	—
n-Propanol	∞	25	—
n-Butanol	7.9	1	0
Aldehydes			
Formaldehyde	∞	13	—
Propionaldehyde	20	10	—
Butyraldehyde	3.7	20	—
Heptaldehyde	Slightly soluble	37	—
Halogenated solvents			
Chloroform	1.0 (15°)	9 ± 4	4 ± 1
Carbon tetrachloride	0.08	17	—
Hydrocarbons			
Benzene	0.08 (22°)	24 ± 4	27
Petroleum ether (35–55°)	—	15 ± 3	8 ± 2

*Values are results of single determinations or, where indicated, averages (with mean deviations) of two separate determinations.
†Gaseous methyl ether was bubbled through reaction mixture.
‡Stimulating activity unaffected by distillation over sodium or by the presence of hydrogen peroxide (0.004%).
§Activity of ethyl ether control, 2.02–2.31 mg. choline/10 min. for spinach, and 2.72–2.84 mg. choline/10 min. for sugar beet.

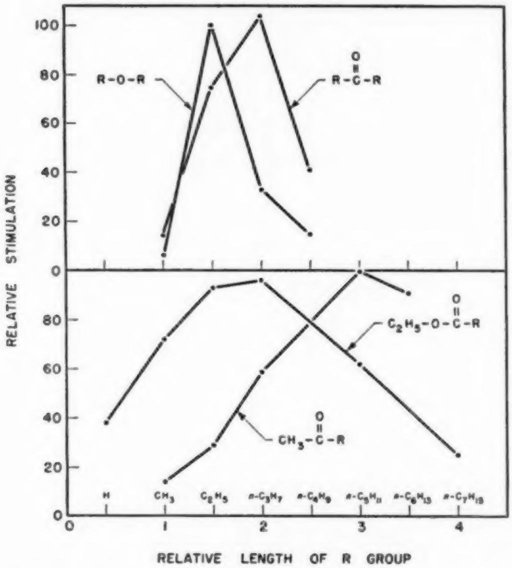


FIG. 1. Influence of length of straight chain hydrocarbon group R on stimulating ability of symmetrical ethers (R—O—R), symmetrical ketones (R—C(=O)—R), methyl ketones (CH_3—C(=O)—R), and ethyl esters (C_2H_5—O—C(=O)—R). Stimulation data taken from Table I (ethyl ether stimulation = 100%); lengths of R groups obtained by measurement of molecular models.

the same number of carbon atoms (Fig. 1), and that butyl acetate produces the same stimulation as ethyl butyrate (Table I). (iii) When the R group is branched, the effect is intermediate between that of the corresponding straight chain group and the straight chain group with one carbon atom less (cf. methyl *i*-propyl ketone with methyl *n*-propyl ketone and methyl ethyl ketone).

Effect of Concentration of Stimulating Solvent

In a previous study of the influence of ethyl ether concentration, at fixed plastid and substrate concentrations, on the rate of choline liberation, it was found that "the rate of choline liberation increased rapidly with increasing concentration of ether to a maximum and constant rate at saturation" (13). Re-examination of the data (Fig. 1, Ref. (13)) revealed, however, that maximum rate was actually reached at concentrations far above saturation, and that the activity – ether concentration curve had a hyperbola-like shape, half-maximum activity being obtained at 0.25 ml. ether per 5.0 ml. reaction mixture. This same value has now been obtained in repeated experiments where the substrate and plastid concentrations were varied over a twofold

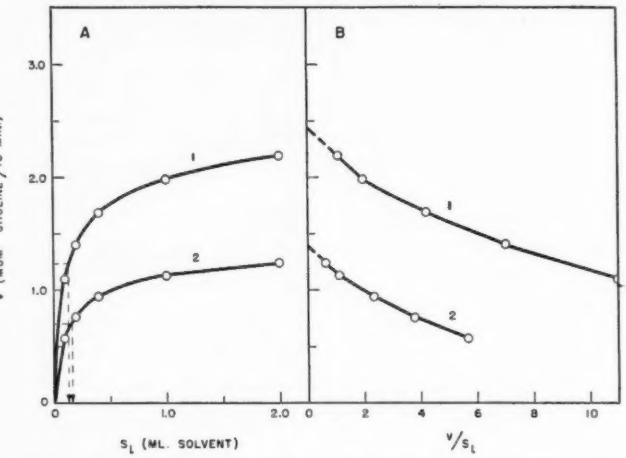


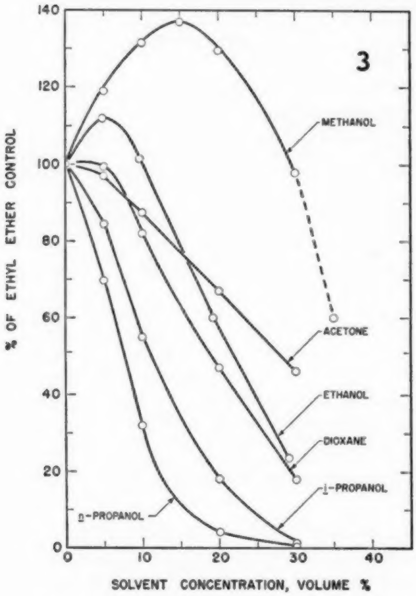
FIG. 2. Dependence of the rate of choline liberation (v) on the amount of ethyl butyrate (S_l) present in the system; A, plot of v against S_l; B, plot of v against v/S_l. Reaction mixtures (5.0 ml.): curve 1, 120 mg. cabbage chloroplasts, 32.2 micromoles of egg lecithin, and acetate buffer (0.1 M, pH 5.5); curve 2, 60 mg. chloroplasts, 16.1 micromoles substrate, and acetate buffer; mixtures were incubated with varying amounts of ethyl butyrate for 10 minutes at 25°.

range. It was desirable, however, to study the concentration effect with a stimulating solvent having a very low solubility in water.

Fig. 2 shows the influence of ethyl butyrate concentration (S_l) on the rate of choline liberation (v) at two concentrations of substrate and plastids, the ratio of substrate to plastids being the same at both concentrations. For both concentration levels, the curves obtained (Fig. 2A, curves 1 and 2) had a hyperbola-like shape. When the data were plotted as v against v/S_l (11), parallel concave curves were obtained (Fig. 2B, curves 1 and 2) rather than straight lines, indicating that the relationship found between v and S_l is not strictly hyperbolic. However, by extrapolating these curves to $v/S_l = 0$, values for v_{max} are obtained which can be used in Fig. 2A to determine the value of S_l for half-maximum activity. The value of S_l producing $\frac{1}{2}\, v_{max}$ was found to be 0.13–0.15 ml., and was independent of substrate and plastid concentrations. This result suggests that the solvent participates directly in one or more intermediate rate-limiting steps, by reversible association with substrate and plastids. Furthermore, the value of S_l for $\frac{1}{2}\, v_{max}$ (0.13–0.15 ml.) is well above the solubility of ethyl butyrate (0.035 ml. per 5 ml. of water), and therefore the observed effects must be due almost entirely to undissolved solvent.

(*b*) *Combination of Solvents*

Fig. 3 shows the variations in ethyl ether stimulation produced by increasing concentrations of alcohols, acetone, or dioxane, which alone produce small stimulations (Table I). Both methanol and ethanol, in the concentration



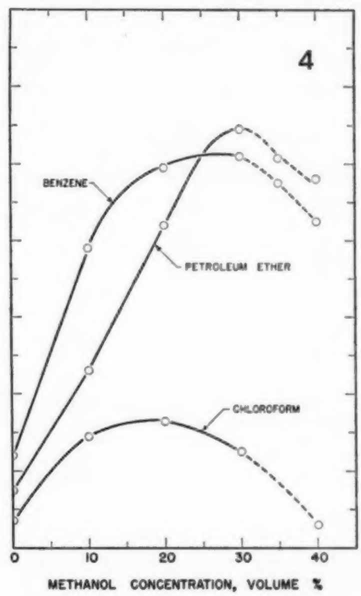


FIG. 3. Effect of water-soluble solvents on stimulation by ethyl ether. Reaction mixtures (5.0 ml.): 100 mg. spinach chloroplasts, 32 micromoles lecithin, water-soluble solvent, and acetate buffer (0.1 *M*, pH 4.7); incubation, 10 minutes with 1 ml. ethyl ether at 25°. Activity of ethyl ether control, 1.91–1.97 mg. choline/10 min. Values for methanol concentrations between 30–40% are unreliable owing to rise in pH of medium to 5.0–5.2.

FIG. 4. Effect of methanol on stimulation by petroleum ether, benzene, and chloroform. Reaction mixtures (see Fig. 3) incubated with 0.5 ml. petroleum ether, 0.1 ml. benzene, or 0.1 ml. chloroform for 10 minutes at 25°. Activity of ethyl ether control, 2.00–2.20 mg. choline/10 min. Values for methanol concentrations between 30–40% are not reliable owing to rise in pH of medium.

ranges 0–30% and 0–10%, respectively, increased the stimulating effect of ether. At higher concentrations these solvents were inhibitory. Increasing concentrations of *n*-propanol or *i*-propanol progressively depressed the ether stimulation to negligible values at 30% concentration. Acetone and dioxane produced little change up to 5% concentration but thereafter progressively depressed the stimulating effect.

It was interesting to determine whether methanol could also increase the stimulating effect of poor stimulators, such as petroleum ether or chloroform. Fig. 4 shows the effect of increasing concentrations of methanol on the stimulating ability of benzene, petroleum ether, or chloroform. The stimulating abilities of both benzene and petroleum ether were increased more than fivefold in the presence of 30% methanol, to values comparable with that of ethyl ether. The stimulating ability of chloroform, however, was increased to a much lesser extent by methanol, a maximum activity of 30% of control being obtained at 20% methanol concentration.

TABLE II

SOLUBILITY OF ETHYL ETHER IN AQUEOUS–ALCOHOL MIXTURES AND DIELECTRIC CONSTANT DATA

Alcohol conc., % by volume	Ether solubility,* S (at 25°) ml./100 ml.	Ratio, $S_{alcohol}/S_{water}$	Dielectric constant, E_0 (27.1°)†		Ratio, $E_{0water}/E_{0alcohol}$	
			Without ether	Saturated with ether	Without ether	Saturated with ether
Methanol						
0	9.0	1.00	77.82	74.53	1.00	1.00
5	9.0	1.00	76.17	72.31	1.02	1.03
10	9.5	1.05	74.33	70.79	1.05	1.05
15	10.5	1.17	—	—	—	—
20	11.5	1.28	70.77	64.27	1.10	1.16
Ethanol						
5	9.0	1.00	75.64	71.57	1.03	1.04
10	9.5	1.05	73.31	69.20	1.06	1.07
15	10.5	1.17	—	—	—	—
20	11.5	1.28	68.95	61.82	1.13	1.20

*Solubility measured by shaking 10.0 ml. of alcohol solution, 0.1 M with respect to sodium acetate buffer (pH 4.7), with increasing amounts of ether until the second phase just appeared.

†Dielectric constants measured by Dr. D. W. Davidson, Division of Applied Chemistry, National Research Council, Ottawa, using a General Radio Model 716-C capacitance bridge, a Model 716-P4 guard circuit, and a stainless steel cell equipped with parallel plate electrodes.

The effects produced by mixtures of methanol and insoluble solvents were not the sums of the stimulations by methanol alone and of those by the insoluble solvents alone, since methanol alone produced only small stimulations (1–12%) over the concentration range 0–30%. Methanol therefore may be considered to have a synergistic effect on the stimulation of ethyl ether, petroleum ether, and benzene. This synergistic effect probably does not result from increase in the water-solubility of the insoluble solvent, nor from decrease in the dielectric constant of the aqueous medium. As shown in Table II, the solubility of ethyl ether in aqueous methanol or ethanol solutions, and the dielectric constants of these media, do not change sufficiently to account for the large increases in ethyl ether stimulation produced by low concentrations of these alcohols (0–10%). Furthermore, while these changes are almost identical for both methanol and ethanol, much greater increases in stimulation are produced by methanol than by ethanol at the same concentration (see Fig. 3).

It is interesting that the first indication of phosphatidase C activity was obtained by Hanahan and Chaikoff (7) when they isolated a phosphatide with a low N/P ratio from carrot root after extraction with an alcohol – ethyl ether mixture. Lecithinase A (8) and lecithinase D (10) have also been shown to operate in an ethyl ether–alcohol medium.

(c) *Surface-active Agents*

The stimulating effect of a number of surface-active substances is given in Table III. Anionic detergents showed the largest effects while cationic and non-ionic detergents, and sterols, showed little or no effect. Since the stimulating abilities of the detergents would be expected to show a dependence on concentration, some of the compounds were investigated further.

TABLE III

STIMULATION OF PLASTID PHOSPHATIDASE C ACTIVITY BY SURFACE-ACTIVE AGENTS

Reaction mixtures (95–100 mg. of spinach chloroplasts, 33 micromoles lecithin, acetate buffer (0.1 *M*, pH 4.7) and a suitable amount of detergent solution or emulsion, in 5.0 ml. total volume) were shaken and incubated for 10 minutes at 25°

Surface-active agent	Final conc. (g./100 ml.)	Relative activation, % ether control§
Non-ionic		
Myrj 45†	0.1	< 1
Span 60†	0.1	5
Atlas G2152*	0.1	12
Cationic		
Cetyl pyridinium chloride*	0.1	0
Anionic		
Sodium dodecyl sulphate*	0.1	31
Sodium taurocholate*	0.2	43
Sodium desoxycholate‡	0.2	19
Sterols		
Stigmasterol†	0.2	1
Sitosterol†	0.2	2
Soybean sitosterols†	0.2	< 1

*In solution.
†In suspension.
‡Forms precipitate of free acid at pH 4.7.
§Activity of ether control, 1.97–2.16 mg. choline/10 min.

Figs. 5 and 6 show the effect of varying the concentrations of sodium dodecyl sulphate (SDS), sodium desoxycholate (SDC), and cetyl pyridinium chloride (CPC), in the absence and in the presence of ethyl ether. In the absence of ether, SDS and SDC produced maximum stimulations of 60–70% of control at 0.02 to 0.025 *M* concentration. In the presence of ether, SDS increased the stimulating effect up to 0.015 *M* concentration, thereafter inhibiting the stimulation; SDC had little effect up to 0.015 *M* concentration, thereafter also inhibiting the ether stimulation (Fig. 5).

Cetyl pyridinium chloride (CPC), however, produced no stimulation whatsoever in the concentration range 3×10^{-4} to 5×10^{-2} *M* and completely depressed the effect of ether at and above 5×10^{-3} *M* concentration (Fig. 6). It is noteworthy that the lecithin was solubilized at concentrations of CPC greater than 3×10^{-2} *M*.

Atlas G2152 produced stimulations of the order of 10% over the concentration range 0.05% to 0.2%, but no stimulation at 0.3%, at which concentration the chloroplasts appeared to be solubilized.

The plant sterols were investigated because of the possibility that they might be the natural stimulating substances present in soybean phosphatide (15). The results obtained, however, eliminate this possibility.

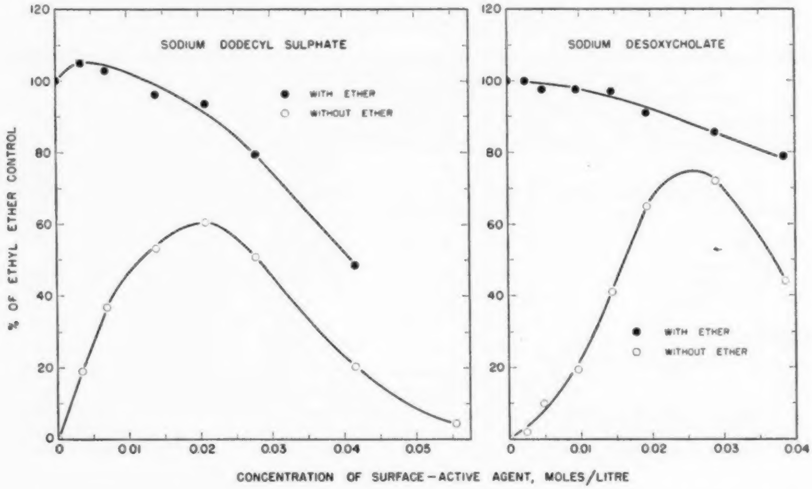


FIG. 5. Effect of sodium dodecyl sulphate and sodium desoxycholate on plastid lecithinase activity. Reaction mixtures (5.0 ml.): 100 mg. spinach chloroplasts, 32 micromoles lecithin, detergent, and acetate buffer (0.1 *M*, pH 4.7); incubation with and without 3 ml. ethyl ether at 25°. Activity of ether control: SDS, 2.11–2.16 mg. choline/10 min.; SDC, 1.84 mg. choline/10 min.

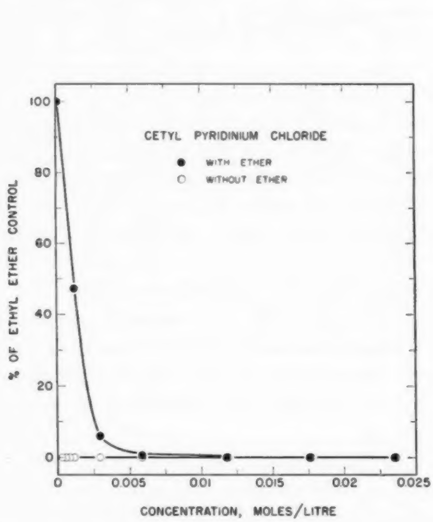


FIG. 6. Effect of cetyl pyridinium chloride on plastid lecithinase activity. Reaction mixtures as in Fig. 5. Activity of ether control, 2.05–2.09 mg. choline/10 min.

Inactivation of Chloroplast Phosphatidase C Activity

(*a*) *Water-soluble Solvents*

The inactivating effects of water-soluble solvents at different concentrations are given in Table IV. Since the presence of some of these solvents in the incubation mixture had been found to affect the activity measurement (Fig. 3), the treated chloroplasts were freed from solvent before incubation with substrate and ethyl ether. All the solvents tested inactivated the chloroplasts completely at high concentrations (60–75%). In the concentration range 0–30%, the relative rates of inactivation by these solvents were in the same order as their inhibiting effects on ethyl ether stimulation (Fig. 3), namely: *n*-propanol > *i*-propanol > dioxane > ethanol > acetone > methanol. Methanol was anomalous in that it produced no inactivation up to 30% concentration, above which it rapidly decreased the activity to zero at 60% concentration. This behavior is consistent with the synergistic effect of methanol on the stimulation by ethyl ether, petroleum ether, and benzene over the concentration range 0–40% (Figs. 3 and 4).

TABLE IV

INACTIVATION OF PHOSPHATIDASE C BY WATER-MISCIBLE SOLVENTS

110 mg. of spinach chloroplasts were stirred at 25° with various solvents at indicated concentrations (total volume, 10 ml.) for 30 minutes, sedimented at 20,000 × g for 5 minutes, and resuspended in 3.5 ml. of H_2O + 0.5 ml. 1 *M* acetate buffer, pH 4.7; incubation with 31 micromoles substrate and 5 ml. ethyl ether for 10 minutes

	Solvent conc., vol. %						
	7	15	25	30	45	60	75
Solvent	% inactivation/30 min.*						
Methanol	—	0	—	0	35	100	100
Ethanol	—	2	—	29	—	83	100
i-Propanol	—	13	—	67	—	91	100
n-Propanol	—	30	—	100	—	100	—
n-Butanol	95†	—	—	—	—	—	—
Acetone	—	0	—	11	—	42	96
Methyl ethyl ketone	—	—	95†	—	—	—	—
Dioxane	—	14	—	36	—	76	99
Tetrahydrofuran	—	—	—	—	—	100	—
Butyrolactone	—	—	—	—	—	99	—
Formaldehyde‡	—	—	88	—	—	—	—

*Activity of untreated control, 2.83–3.06 mg. choline/10 min. Per cent inactivation

$$= 100 \left[1 - \frac{\text{activity of treated chloroplasts}}{\text{activity of untreated control}}\right].$$

†Saturated with solvent.
‡Concentration of formaldehyde is on weight basis.

TABLE V

INACTIVATION OF PHOSPHATIDASE C BY WATER-INSOLUBLE SOLVENTS

Mixtures of 100–107 mg. spinach chloroplasts, 0.5 ml. 1 *M* acetate buffer (pH 4.7), and water to 4.5 ml. were shaken with 1 ml. of solvent for 10 or 30 minutes at 25°, then incubated with 0.5 ml. of 5% lecithin suspension and 5 ml. of ethyl ether (except for highly stimulating solvents) for 10 minutes. Controls run in the same way, except treatment with solvent was reduced to 0.5 minutes

Solvent	% inactivation*	
	10 min. treatment	30 min. treatment
Ethyl ether	11 ± 4†	30 ± 14†
i-Propyl ether	5	21
n-Propyl ether	13	53
n-Butyl ether	0	0
Di-*n*-propyl ketone‡	2	8
Cyclopentanone	—	98
Ethyl butyrate‡	3	6
Chloroform	12	22
Carbon tetrachloride	0	0
Benzene	8	19
Petroleum ether (b.p. 35–55°)	0	5

*Per cent inactivation $= 100\left[1-\dfrac{\text{activity of treated chloroplasts}}{\text{activity of control}}\right]$.

†Averages, with mean deviations, of four separate determinations.

‡No ether was added during incubation: stimulation achieved by solvent itself (2–2.5 ml.).

It is noteworthy that, of the solvents tested, *i*-propanol and *n*-propanol were the most damaging to the chloroplast phosphatidase activity and were also poor stimulators (Table I). These solvents should prove advantageous for extraction of plant phosphatides without accompanying enzymatic degradation.

(*b*) *Water-insoluble Solvents*

The inactivating effects of water-insoluble solvents are given in Table V. With the exception of cyclopentanone, these solvents inactivated the enzyme at a relatively slow rate (1–2% per minute or less); some solvents, e.g. *n*-butyl ether, carbon tetrachloride, and petroleum ether had no effect over the period studied. Large variations in the inactivations produced by ethyl ether were encountered, for which no explanation was apparent.

No direct relationship appeared to exist between the stimulating ability of a solvent and its inactivating effect. While all of the highly stimulating solvents (ethyl ether, propyl ketone, and ethyl butyrate) produced relatively small inactivations, some low-stimulating solvents, such as petroleum ether or chloroform, also produced small inactivations, and others, such as cyclopentanone and water-soluble solvents, were highly inactivating.

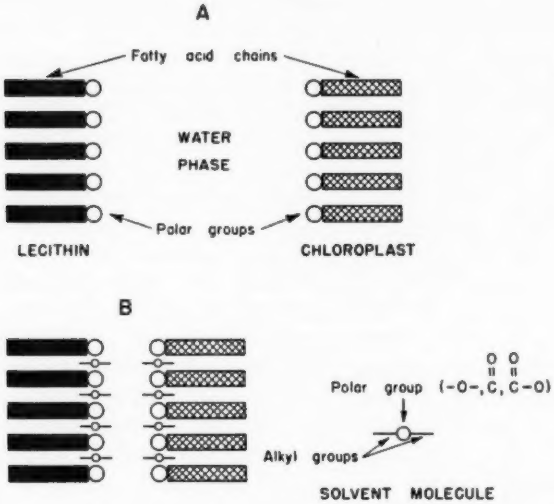


FIG. 7. Mechanism of stimulation of plastid phosphatidase C activity by solvents. A, orientation of lecithin molecules at a micelle surface, and of phospholipids at the chloroplast surface; B, orientation of linear aliphatic ether, ketone, or ester molecules at lecithin and plastid surfaces.

Discussion

The foregoing results show that ethyl ether is not unique in its ability to stimulate chloroplast phosphatidase C activity. The compounds producing large stimulations may be grouped as follows: (i) solvents of linear structure possessing a disubstituted oxygen functional group to which hydrocarbon chains of appropriate length are attached, e.g., linear aliphatic ethers, ketones, or esters; (ii) mixtures of methanol with ethyl ether, petroleum ether, or benzene; and (iii) substances containing an anionic group attached to a long chain or steroidal hydrocarbon group, e.g., sodium alkyl sulphates or bile salts. It is important to note that all these substances produce very little inactivation of the phosphatidase C activity under conditions in which they stimulate the reaction.

One may now consider the mechanism whereby stimulation is achieved by these substances. In another paper (16), it is shown that all stimulating solvents cause the plastids and substrate particles to coalesce and that the reaction actually takes place in the coagulum. The solvent might produce coalescence indirectly by changing the dielectric constant of the suspending medium, or directly by forming adsorption complexes with the particles. Direct participation of the stimulating solvent is strongly suggested by the results shown in Fig. 2. The fact that the amount of solvent producing half-maximum activity is the same for two different concentrations of substrate and plastids indicates that the solvent participates in one or more intermediate

rate-limiting steps, involving reversible association of solvent with substrate and plastids. Strong adsorption of ethyl ether vapor by monolayers of lecithin has recently been reported (5); during the course of this work, chloroplast particles have been observed to adsorb and retain ether very strongly (see also Fig. 5 in Ref. (16)). The formation of adsorption complexes between the stimulating solvent and lecithin or chloroplasts, and the resultant coalescence of substrate and plastids may be pictured as follows:[3]

Lecithin molecules in aqueous medium are probably arranged in micelles, in which the polar (hydrophilic) groups are oriented towards the water phase and the fatty acid chains are directed away from the water phase and as close together as possible (1), as shown in Fig. 7A. The chloroplast fragments contain phospholipids (20, 22) which may be arranged at the surfaces in a similar manner (Fig. 7A). Both lecithin and plastid particles would therefore preferentially attract water molecules and would not tend to coalesce.

If the surfaces of the lecithin and plastid particles could be made lipophilic they would attract each other more strongly than water, and coalescence should occur. It is suggested that the stimulating solvents (linear aliphatic ethers, ketones, or esters) are adsorbed at the surfaces of the particles, as shown in Fig. 7B: the polar group in the solvent molecule (ether or carbonyl group) would be attracted to the polar groups at the surfaces of the particles, whereupon one of the hydrocarbon groups attached to the solvent polar group would penetrate the surface, and the other would project outwards, making the surface sufficiently lipophilic for coalescence to occur.

This hypothesis would explain why only those solvents having two linear hydrocarbon groups attached to an oxygen functional group can produce large stimulations. Furthermore, it follows that alcohols, aldehydes, and cyclic ethers, ketones, or esters are ineffective, because they would penetrate the surface with their hydrocarbon groups directed towards the interior of the particles and their polar groups towards the water, thus leaving the surface hydrophilic. Hydrocarbons are also ineffective since they either are not strongly attracted to the surface, or if they are, will penetrate into the interior of the particles.

The mechanism outlined also accounts, qualitatively, for the observed dependence of the stimulation by linear ethers, ketones, or esters on the size of the hydrocarbon groups attached to the oxygen functional group (Fig. 1). If the hydrocarbon groups (R, in $R{-}O{-}R$ and $R{-}\overset{\overset{O}{\|}}{C}{-}R$) are both short, the penetrated surface will still not be sufficiently lipophilic, and if the groups are very long, both will penetrate into the interior still leaving the surface hydrophilic. Thus, the mechanism predicts that a maximum stimulation should be achieved with symmetrical ethers or ketones when the R group

[3]The surface-penetration mechanism presented is based on ideas suggested to the author (personal communication May 18, 1955) by Dr. J. T. Davies, King's College, London, who predicted correctly that diethyl ketone and ethyl propionate would show effects similar to that of ethyl ether. For further reference, see some recent publications by Davies (2, 3, 4).

has an appropriate length (Fig. 1). Similar considerations apply also to unsymmetrical ketones and to ethyl esters. Furthermore, with esters, it should be immaterial whether a long chain is attached to the carbonyl carbon and a short one to the ester oxygen, or vice versa. The mechanism would predict that ethyl butyrate and butyl acetate produce exactly the same stimulation, and this is precisely what has been observed experimentally (Table I).

The synergistic effect of methanol on the stimulation by ethyl ether, petroleum ether, or benzene (Figs. 3 and 4) may also be explained by the surface-penetration mechanism. Perhaps methanol is first adsorbed at the surfaces, making them sufficiently lipophilic to attract more of the stimulating solvent than would be possible in absence of methanol.

The stimulation by anionic detergents probably occurs by means of a somewhat different mechanism. The concentration range (0.02–0.025 *M*) in which these detergents produce maximum stimulation is in or above the critical region for micelle formation (21). Although maximum "solubilization" of chloroplasts by 0.02 *M* sodium dodecyl sulphate at pH 8.9 has been observed (17), this did not occur at the pH (4.7) used in the present studies. Instead, at 0.02 *M* concentration of anionic detergents, the plastids and substrate were observed to coalesce and rise to the surface. ("Solubilization" did occur with 0.3% Atlas G2152, but enzymatic activity was no longer detectable.) It is possible that the anionic detergents produce coalescence of substrate and plastids through neutralization of the net positive charges (due to the quaternary nitrogen group of the choline residue) on the surfaces of the particles by the negatively charged anionic detergent micelles. This mechanism would also account for the ineffectiveness of cationic and non-ionic detergents (Table III). Furthermore, the observed dependence of stimulation on the anionic detergent concentration (Fig. 5) is very similar to the dependence of mutual coagulation of two colloids of opposite charge (e.g., gelatin and gum arabic at pH 3) on their relative concentrations (see (19)).

Acknowledgment

The author is indebted to Dr. J. T. Davies for valuable advice concerning the mechanism of solvent stimulation, and to Dr. D. W. Davidson for dielectric constant measurements. The excellent technical assistance of Miss Fay Inglis is acknowledged.

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PHOSPHOLIPID METABOLISM IN RAT LIVER SLICES

EFFECT OF HYPOPHYSECTOMY AND ADRENALECTOMY ON THE LABELLING OF PHOSPHOLIPIDS WITH ACETATE-1-C^{14},[1]

DOROTHY KLINE[2] AND R. J. ROSSITER

Abstract

In rats fed ad libitum the labelling of the acetone-insoluble lipids (phospholipids) of liver slices respiring in the presence of acetate-1-C^{14} was decreased 2 days after the removal of either the pituitary or the adrenal glands. The effect was not observed 4 days after hypophysectomy. Moreover, the effect was not observed with liver slices from hypophysectomized or adrenalectomized animals that were either deprived of food for the 12 hours immediately prior to killing, or force-fed with glucose 4 hours before killing. Hypophysectomy caused a decrease in the labelling of the fatty acids in the acetone-soluble fraction remaining in solution after precipitation of the phospholipids. This decrease was observed both in liver slices from animals fed ad libitum and in those from fasted (12 hours) animals. In rats fed ad libitum the labelling of the non-esterified cholesterol of the liver slices was decreased by hypophysectomy, but not by adrenalectomy. It is concluded that the decrease in the labelling of the acetone-insoluble lipids in liver slices from operated rats was primarily the result of a change in the nutritional status of the operated animals. The data are insufficient to permit any conclusions concerning the cause of the decrease in labelling of either the fatty acids of the acetone-soluble lipids, or the non-esterified cholesterol.

Introduction

Pihl and Bloch (14) showed that the acetone-insoluble lipids (phospholipids) of rat liver slices respiring in a suitably buffered medium containing acetate-1-C^{14} were labelled metabolically. This finding was confirmed by Kline and DeLuca (8), who studied the labelling in some detail. The present paper describes the effect of removing the pituitary or adrenal glands on the subsequent labelling of the acetone-insoluble lipids of liver slices. At the same time, some observations were made on the labelling of the free or non-esterified cholesterol, and on the labelling of the fatty acids from the acetone-soluble fraction remaining after precipitation of the phospholipids.

Methods

Male rats of the Sprague–Dawley strain (150–200 g.) were used throughout. Hypophysectomized rats were obtained from Hormone Assay Laboratories, Inc., Chicago. Macroscopic examination of the pituitary region and microscopic examination of the same region in representative animals failed to reveal any evidence of remaining pituitary tissue. The operated rats showed the adrenal atrophy typical of hypophysectomy. Both the hypophysectomized rats and corresponding controls, obtained from the same source, were supplied with a diet consisting of vitamin-enriched bread, oranges, and milk.

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Adrenalectomy was performed in the laboratory. Both adrenalectomized rats and corresponding controls were supplied with Master Fox Cubes (Toronto Elevators, Ltd.). The adrenalectomized rats received drinking water containing 1% sodium chloride. Unless otherwise stated, both hypophysectomized and adrenalectomized rats were studied 2 days after operation.

Slices of liver tissue prepared from the operated and corresponding control rats of the same strain, sex, and age were incubated for 4 hours in a Krebs–Ringer bicarbonate medium containing acetate-1-C^{14}. At the end of the incubation, the lipids were separated and the specific activity of the desired lipids determined. The methods of killing, perfusion of liver, cutting of slices, incubation with acetate-1-C^{14}, and the extraction, separation, purification, and counting of the lipids have been described previously (8).

Results

Phospholipids

Table I shows that in rats fed ad libitum removal of the pituitary gland caused a decrease in the subsequent labelling of the phospholipids of liver slices when the experiments were carried out 2 days after the operation. When the experiments were carried out 4 days after the operation, the mean specific activity of the phospholipids was less, but the decrease was not significant statistically. Table I also shows that removal of the adrenal glands caused a similar decrease in the labelling of the phospholipids in experiments carried out 2 days after the operation.

TABLE I

EFFECT OF HYPOPHYSECTOMY AND ADRENALECTOMY ON THE LABELLING OF PHOSPHOLIPIDS OF LIVER SLICES OF RATS FED AD LIBITUM

Expt. No.	Operation	Days after surgery	Controls	Operated	*P*
1	Hypophysectomy	2	5,750 ± 660* (6)	1,170 ± 250 (6)	< 0.001
2	Hypophysectomy	2	3,520 ± 650 (6)	1,170 ± 340 (6)	< 0.01
3	Hypophysectomy	4	5,700 ± 140 (6)	3,640 ± 1,400 (6)	> 0.2
4	Hypophysectomy	4	2,260 ± 770 (6)	1,900 ± 880 (7)	> 0.9
5	Adrenalectomy	2	4,820 ± 330 (6)	1,000 ± 260 (8)	< 0.001
6	Adrenalectomy	2	5,430 ± 98 (5)	3,050 ± 210 (5)	< 0.05

*Specific activity (counts/min./mg.). Mean ± standard error of mean. The figures in parentheses indicate the number of animals.

Because of the well-known decrease in the labelling of total fatty acids in liver slices of animals deprived of food prior to the experiment (3, 9, 10), and of the similar findings for total cholesterol (18), the possibility was considered that the effects reported in Table I might be due to the nutritional status of the animal, rather than due to the lack of the hormones normally produced by the glands removed. To test this hypothesis, the experiments described in Tables II and III were performed.

From Table II it can be seen that there was no significant decrease in the labelling of the phospholipids in liver slices from either hypophysectomized or adrenalectomized rats when the animals were deprived of food for the 12 hours immediately prior to killing. If the figures for the control animals are compared with those presented in Table I for animals fed ad libitum, it can be seen that there was a great decrease in the labelling of the phospholipids brought about by withholding food for the 12 hours preceding death. This finding has already been commented upon briefly (17); it will form the subject of a future report.

TABLE II

EFFECT OF HYPOPHYSECTOMY AND ADRENALECTOMY ON THE LABELLING OF PHOSPHOLIPIDS OF LIVER SLICES OF FASTED (12 HOURS) RATS

Operation	Control	Operated	P
Hypophysectomy	390 ± 14* (3)	400 ± 42 (6)	> 0.9
Adrenalectomy	560 ± 43 (5)	430 ± 57 (6)	> 0.3

*Specific activity (counts/min./mg.). Mean ± standard error of mean. The figures in parentheses indicate the number of animals.

TABLE III

EFFECT OF FORCE FEEDING ON THE CHANGES IN THE LABELLING OF PHOSPHOLIPIDS OF RAT LIVER SLICES

Treatment	Control	Operated	P
Hypophysectomized—fed ad libitum	6,170 ± 1,290* (6)	2,210 ± 470 (4)	< 0.001
Hypophysectomized—force-fed	7,710 ± 2,400 (6)	5,530 ± 1,800 (6)	> 0.8
P	> 0.5	< 0.01	
Adrenalectomized—fed ad libitum	4,150 ± 280 (6)	1,610 ± 420 (6)	< 0.001
Adrenalectomized—force-fed	6,740 ± 790 (6)	4,970 ± 770 (6)	> 0.1
P	< 0.02	< 0.01	

*Specific activity (counts/min./mg.). Mean ± standard error of mean. The figures in parentheses indicate the number of animals.

Table III shows the results of further experiments with rats fed ad libitum. Again, removal of either the pituitary or the adrenal glands was found to cause a decrease in the labelling of the phospholipids of the liver slices, thus confirming the findings presented in Table I. Comparable animals were given 1.5 g. glucose (5 ml. of a 30% solution) by stomach tube 4 hours before killing. In such force-fed animals there was no significant decrease in the labelling of the phospholipids of the liver slices after removal of either the pituitary or the adrenal glands. The force feeding caused only a slight increase in the labelling of the phospholipids in the control animals, but in both the hypophysectomized and the adrenalectomized animals the force feeding caused a highly significant increase in the labelling of the phospholipids in the liver slices.

Fatty Acids from Acetone-soluble Lipids

Table IV shows that hypophysectomy caused a decrease in the labelling of the fatty acids remaining in solution after acetone precipitation of the phospholipids. This decrease was significant in the liver slices from rats that were fed ad libitum and, in contrast with the findings reported for the phospholipids (Table II), the decrease was still statistically significant in the liver slices from fasted animals.

Table IV also shows that withholding food for 12 hours caused a great decrease in the labelling of the fatty acids of the acetone-soluble lipids. In this regard, the fatty acids of the acetone-soluble lipids resemble the total fatty acids (3, 9, 10), the phospholipids (cf. Tables I and II), and total cholesterol (18).

Non-esterified Cholesterol

It can be seen from Table V that in rats fed ad libitum removal of the pituitary caused a great decrease in the subsequent labelling of the free or non-esterified cholesterol in the liver slices. In contrast, removal of the adrenals caused no such significant change in the labelling of the free cholesterol.

TABLE IV

EFFECT OF HYPOPHYSECTOMY ON THE LABELLING OF FATTY ACIDS FROM ACETONE-SOLUBLE LIPIDS OF RAT LIVER SLICES

Expt. No.	Nutritional status	Control	Hypophysectomized	P
1	Fed ad libitum	3,070 ± 370* (6)	760 ± 26 (6)	< 0.001
2	Fed ad libitum	5,620 ± 1,260 (6)	280 ± 110 (6)	< 0.001
3	Fasted (12 hr.)	510 ± 74 (3)	42 ± 5 (6)	< 0.001

*Specific activity (counts/min./mg.). Mean ± standard error of mean. The figures in parentheses indicate the number of animals.

TABLE V

EFFECT OF HYPOPHYSECTOMY AND ADRENALECTOMY ON THE LABELLING OF NON-ESTERIFIED CHOLESTEROL OF LIVER SLICES OF RATS FED AD LIBITUM

Expt. No.	Operation	Days after surgery	Control	Operated	P
1	Hypophysectomy	2	50,500 ± 4,700* (6)	8,100 ± 3,100 (6)	< 0.001
2	Hypophysectomy	2	139,000 ± 14,000 (6)	6,200 ± 1,700 (6)	< 0.001
3	Hypophysectomy	4	92,900 ± 17,000 (6)	26,900 ± 9,300 (6)	< 0.01
4	Hypophysectomy	4	33,500 ± 4,500 (6)	12,200 ± 4,700 (7)	< 0.01
5	Hypophysectomy	4	58,300 ± 7,300 (6)	15,000 ± 9,200 (4)	< 0.01
6	Adrenalectomy	2	137,000 ± 12,000 (6)	119,000 ± 14,000 (8)	> 0.7
7	Adrenalectomy	2	158,000 ± 17,000 (5)	185,000 ± 10,000 (5)	> 0.2
8	Adrenalectomy	4	137,000 ± 24,000 (6)	121,000 ± 26,000 (6)	> 0.7

*Specific activity (counts/min./mg.). Mean ± standard error of mean. The figures in parentheses indicate the number of animals.

Discussion

Phospholipids

Two days after removal of either the pituitary or adrenal glands there was a decrease in the labelling of acetone-insoluble lipids (phospholipids) of liver slices from rats fed ad libitum. The finding that there was no significant decrease in the labelling of the phospholipids in liver slices from hypophysectomized animals studied 4 days after removal of the pituitary suggested that the change observed in the operated animals might be due to the poor nutritional status of such animals. The fact that no decrease was observed in experiments in which both the control and the operated animals were deprived of food for the 12 hours immediately prior to killing (Table II), or in which both control and operated animals received large quantities of glucose by stomach tube (Table III), is good evidence in favor of this view.

A short time after the removal of the pituitary from a rat, the metabolic condition of the liver is not good. For instance, Ringler and Leonard (16) reported that there was a decrease in the concentration of coenzyme A in the liver of hypophysectomized rats. Moreover, Chernick and Moe (5) showed that this decrease could not be observed at longer time intervals after the operation.

It would appear, therefore, that the poor nutritional status of the operated animals, probably associated with a decreased food consumption, was responsible for the results obtained. A number of experiments, not reported, were done with controls subjected to a sham operation. In general, the

degree of labelling observed in these sham-operated animals was similar to that reported for the operated animals, but the variability in the results was much greater.

For the short-term experiments reported here, the running of pair-fed controls was not practicable. However, dietary restriction, as is necessary in pair feeding, was found to cause a great reduction in the labelling of the phospholipid. This effect, in normal, hypophysectomized and adrenalectomized animals, forms the subject of a report now in preparation.

Pihl and Bloch (14) reported that the fatty acid portion of the phospholipid molecule was labelled in liver slices respiring in the presence of acetate-1-C^{14}. Pritchard (15) has confirmed this finding and has shown that, when liver slices were incubated with acetate-1-C^{14}, neither the glycerol nor the nitrogen-containing moiety of the phosphoglyceride molecule was labelled to any significant extent.

Because the fatty acids of phospholipids contribute greatly to the total fatty acids of the liver, the present findings are of some interest in relation to the conflicting reports on the effect of hypophysectomy on the labelling of the total fatty acids in liver slices. It has been widely accepted that hypophysectomy enhances the incorporation of labelled acetate into the total fatty acids of rat liver slices (4, 7). However, a review of the literature revealed that this belief is founded upon a minimum of experimental evidence. Brady, Lukens, and Gurin (4), as the result of experiments with a small number of animals, concluded that hypophysectomy caused an increase in the subsequent labelling of the total fatty acids of liver slices from acetate-1-C^{14}. These workers also reported that the prior administration of growth hormone to Houssay cats caused a decrease in the subsequent labelling of the total fatty acids of liver slices incubated with acetate-1-C^{14}. Perry and Bowen (12) showed that previous injection of growth hormone to rats caused a decrease in the labelling of the total fatty acids of liver slices from acetate-2-C^{14}, while in the intact rat Welt and Wilhelmi (19) showed that growth hormone caused a decrease in the uptake of deuterium oxide into the total fatty acids of the liver. On the other hand, Baruch and Chaikoff (2) reported that hypophysectomy caused a decrease in the labelling of total fatty acids from acetate-1-C^{14} in liver slices from rats fed ad libitum. Our experiments, both with the acetone-insoluble lipids and with fatty acids from the acetone-soluble lipids, indicate that, under conditions of ad libitum feeding, the labelling of fatty acids was decreased by removal of the pituitary. This decrease was not observed in fasted or force-fed animals, but in no case was an increase in labelling observed in liver slices from hypophysectomized rats.

With regard to adrenalectomy, a clear picture is similarly lacking. Welt and Wilhelmi (19) found that with intact rats adrenalectomy caused an increase in the uptake of deuterium oxide into the total fatty acids of the livers, whereas Perry and Bowen (11–13) reported a decrease in the labelling of total fatty acids of liver slices incubated with acetate-2-C^{14}. However, in

contrast with the findings reported here for phospholipids, the decrease in the labelling of the total fatty acids in adrenalectomized animals was found to persist in the livers from both fasted and force-fed rats (11–13).

Prior administration of cortisone would appear to cause a decrease in the labelling of total fatty acids in liver slices (4, 7), i.e. a change in the same direction as that produced by adrenalectomy. On the other hand, Altman, Miller, and Bly (1) reported that in perfused rat liver the addition of cortisone to the perfusion fluid enhanced the incorporation of acetate-1-C^{14} into the fatty acids of the phospholipids and triglycerides. Our experiments suggest that, for phospholipids at any rate, the decreased labelling brought about by removal of the adrenal glands, like that attributable to the removal of the pituitary, is primarily the result of a change in the nutritional status of the animal.

Fatty Acids from Acetone-soluble Lipids

The finding that removal of the pituitary caused a decrease in the subsequent labelling of the fatty acids remaining in solution after the precipitation of the phospholipids (Table IV) is consistent with the report of Baruch and Chaikoff (2) for total fatty acids. The decreased labelling of the fatty acids of the acetone-soluble lipids was observed both in animals fed ad libitum and in fasted animals. It will be recalled that in fasted animals there was no such decrease in the labelling of the phospholipids.

Non-esterified Cholesterol

Gould and Taylor (6) found that in slices of dog and rabbit liver incubated in the presence of labelled acetate, most of the radioactivity was present in the free rather than in the esterified cholesterol, an observation we have confirmed for rat liver slices. For this reason, the free, i.e. the most recently formed, cholesterol was investigated in preference to the total cholesterol, which has been studied in most work of this type (13, 18).

The finding that in rats fed ad libitum the labelling of free cholesterol was decreased by hypophysectomy (Table V) is consistent with the report of Tomkins, Chaikoff, and Bennett (18) for total cholesterol. Adrenalectomy was found to cause no significant change in the labelling of the free cholesterol. This is in contrast with the labelling of the phospholipids, where in rats fed ad libitum the decrease brought about by removal of the adrenal glands was more consistent than that brought about by removal of the pituitary.

In rats fed ad libitum, Perry and Bowen (13) were unable to demonstrate that adrenalectomy caused a significant change in the labelling of total cholesterol from acetate-2-C^{14} in liver slices incubated in a bicarbonate buffer. However, when the bicarbonate buffer of the incubating medium was replaced with phosphate buffer, a decrease in the labelling of total cholesterol was observed. The significance of this buffer effect is difficult to assess at the present time.

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METABOLISM OF DL-3,4-DIHYDROXYPHENYLALANINE-α-C^{14} IN BOVINE ADRENAL HOMOGENATE[1]

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Abstract

The metabolism of DL-3,4-dihydroxyphenylalanine-α-C^{14} (DL-DOPA) by beef adrenal medulla homogenates has been investigated. Following incubation seven radioactive zones appeared on the chromatograms. Five of these substances have been identified using admixture chromatography: DOPA, DOPA – pyridoxal phosphate Schiff base, dopamine, noradrenaline, and 3,4-dihydroxyphenylacetic acid. Conversion of DL-DOPA to noradrenaline varied from 5 to 14% when calculated on the initial radioactivity of the DOPA incubated. No radioactive adrenaline, 3,4-dihydroxyphenylserine, isoprenaline, homovanillic acid, or epinine was detected.

Introduction

In 1939, Blaschko (1) suggested that the decarboxylation of DOPA was an important step in the biogenesis of adrenaline and noradrenaline. Consequently the adrenal glands of several species were investigated for DOPA decarboxylase activity. This enzyme was first demonstrated in the bovine adrenal medulla by Langemann (8). Furthermore, dopamine, the metabolite resulting from this reaction, was detected in the adrenal gland of ox and sheep using paper chromatography (12).

According to Vinet (13), formation of adrenaline takes place during incubation of dopamine with suprarenal tissue. However, the colorimetric estimation used was not specific for adrenaline and consequently the resulting catecholamine could have been noradrenaline. We have previously reported that no radioactivity was present in the adrenaline and noradrenaline of the adrenal glands of the albino rat after administration of DL-DOPA-α-C^{14} (10). Demis, Blaschko, and Welch (3) have detected radioactive dopamine and noradrenaline following incubation of DL-DOPA-α-C^{14} in the presence of bovine adrenal medulla homogenate. Furthermore Hagen (5) has shown that noradrenaline arises from dopamine in the presence of chicken adrenal homogenates.

In an attempt to elucidate the participation of DOPA in the biosynthesis of adrenaline and noradrenaline, the behavior of DL-DOPA-α-C^{14} in the presence of beef adrenal medulla homogenate was studied. Paper chromatography was employed to detect and identify the radioactive metabolites. Although particular attention was paid to adrenaline and noradrenaline other metabolites were also identified.

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Contribution from the Laboratoire de Physiologie, Université de Montréal, Montreal, Quebec.
[2]Predoctoral Fellow of the J. Rhéaume Foundation.

Experimental

Materials

DL-3,4-DOPA-α-C^{14} was purchased from Nuclear, Chicago. It had a specific activity of 5.5×10^6 counts per minute per milligram and was chromatographically pure. 3,4-Dihydroxyphenylacetic acid was kindly supplied by Dr. C. W. Murray of the U.S. Agricultural Research Service, Albany, Calif., and homovanillic acid by Dr. K. N. F. Shaw of the Laboratory for the Study of Hereditary and Metabolic Disorders, University of Utah, Salt Lake City, Utah. 3,4-Dihydroxyphenylserine was kindly furnished by Dr. H. Blaschko. Noradrenaline, adrenaline, epinine, isoprenaline, and dopamine were the commercial products.

Methods

Beef adrenal glands were obtained from the slaughterhouse as soon as possible after the animal's death. The medullary portion was dissected out and homogenized in the presence of isotonic potassium chloride for 1 minute in a Waring blendor at 0° C. It was calculated that from 30 to 60 minutes elapsed between the animal's death and the preparation of the homogenate. The incubations were carried out in Warburg flasks for 2 hours at 37° C. A typical incubation medium contained the following: 2 ml. of the medullary homogenate (25% w/v), 0.4 ml. of 0.07 *M* phosphate buffer pH 7.4, 0.1 ml. of 0.005 *M* DL-DOPA-α-C^{14}, 0.2 ml. of 2 *N* sodium hydroxide in the center well.

Following incubation, the reaction was stopped by addition of 0.2 ml. of concentrated hydrochloric acid. After centrifugation the protein precipitates were washed twice with distilled water. The combined supernatants were adjusted to pH 3 with 1 *N* sodium hydroxide and evaporated to dryness *in vacuo* at room temperature. The dry residues were taken up in 0.5 ml. of 0.001 *N* hydrochloric acid and centrifuged to remove any insoluble residue.

The extracts were chromatographed on paper using the descending technique. All aliquots were routinely developed in three solvent systems. Solvent A consisted of benzyl alcohol:acetic acid:water, 5:1:1; solvent B, *n*-butanol: 1 *N* hydrochloric acid, 4:1 (6); and solvent C, *n*-butanol:acetic acid:water, 7:1:2 (7). Chromatography was carried out on Whatman No. 1 paper at room temperature. The general procedure was to apply on the starting line, 4 cm. apart, two identical spots both containing an aliquot of the tissue extract plus one or several control substances.

Upon completion of the chromatographic separation, the papers were dried overnight in a fume cupboard at room temperature and cut vertically in two parts. The first half was sprayed with the potassium ferricyanide – ferric sulphate reagent of Goldenberg *et al.* (4) to localize the position of the control substances. The second half was cut horizontally in 1 cm. strips. These strips were eluted with 0.01 *M* hydrochloric acid. The eluates were collected in cupped planchets and evaporated to dryness, and their radioactivity was measured in a gas flow counter.

Results

R_f Values of Control Substances

The control substances suspected of being metabolites of DOPA were chromatographed in the three solvents described. These componds were spotted from 0.001 *N* hydrochloric acid solution and their R_f values are listed in Table I. DOPA and adrenaline were found to overlap in solvent B; however, they separated in solvent A and C. Although adrenaline and dopamine migrated at identical rates in solvent A, either solvent B or C afforded a resolution of these two substances.

TABLE I

R_f VALUES OF CONTROL SUBSTANCES

Compounds	Solvent A BzOH: acetic acid: water (5:1:1)	Solvent B BuOH: 1 *N* HCl (4:1)	Solvent C BuOH: acetic acid: water (7:1:2)
3,4-DOPA – pyridoxal phosphate Schiff base	0.08	0.05	0.04
3,4-Dihydroxyphenylserine	0.08	0.07	0.08
3,4-DOPA	0.17	0.18	0.20
Noradrenaline	0.25	0.12	0.29
Adrenaline	0.43	0.17	0.34
Dopamine	0.43	0.23	0.42
Epinine	0.59	0.28	0.45
Isoprenaline	0.62	0.37	0.54
3,4-Dihydroxyphenylacetic acid	0.72	0.71	0.76
Homovanillic acid	0.90	0.80	0.88

According to Schott and Clark (11), DOPA reacts nonenzymatically with pyridoxal phosphate to give a Schiff's base complex. The R_f value of this complex was determined in the present study by assuming its identity with the single new radioactive peak which appeared after a similar incubation of DL-DOPA-α-C^{14}.

Distribution of the Radioactivity in the Incubation Extracts

The radioactivity present in the extracts of incubated adrenal homogenates was distributed among seven peaks in solvent B and six peaks in solvents A and C. A typical distribution of radioactive metabolites characteristic of 15 incubations is shown in Fig. 1. In control experiments where DL-DOPA-α-C^{14} was incubated in the absence of adrenal homogenate, only one radioactive spot was observed. This spot migrated in positions analogous to that of DOPA in the three solvents.

Five of the radioactive spots have been tentatively identified by admixture chromatography using the three solvent systems. Thus it was noted that the principal radioactive substance (peaks IIC, IIIA, IIIB) was radioactive DOPA. This recovered DOPA generally accounted for about 50 to 60% of the starting material. The second largest spot (peaks VA, IVB, IVC) had migrating properties identical with that of dopamine. In most cases 10 to 15% of the original radioactivity was present in the dopamine zones. In butanol: 1 *N* HCl dopamine did not entirely separate from DOPA. However complete separation was achieved with the two other systems and the yields

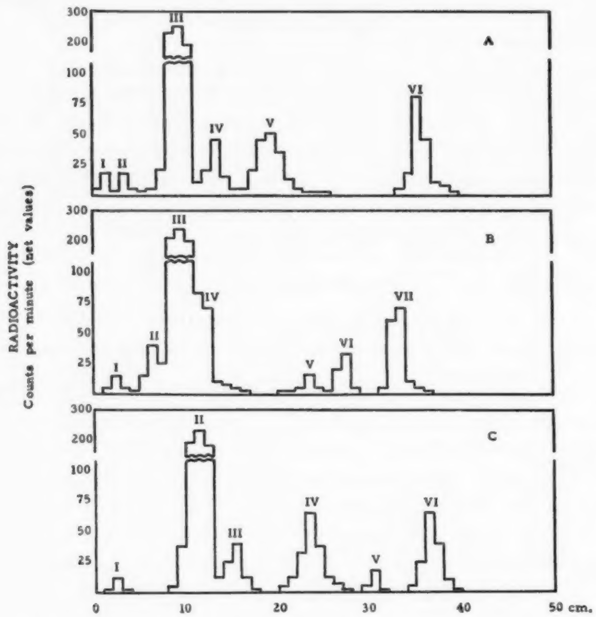

FIG. 1. Paper chromatography of incubation extracts.
A, Solvent system: benzyl alcohol: acetic acid: water (5:1:1), 30 hours.
B, Solvent system: *n*-butanol: 1 *N* HCl (4:1), 35 hours.
C, Solvent system: *n*-butanol: acetic acid: water (7:1:2), 26 hours.

reported are estimated from these chromatograms. Another peak of the same magnitude, i.e. 10%, was also found in all cases. The position of this peak coincides in the three solvent systems with that of 3,4-dihydroxyphenylacetic acid (peaks VIA, VIIB, VIC). Noradrenaline could also be detected in this manner and generally contained from 5 to 14% of the original activity (peaks IVA, IIB, IIIC). The two or three remaining spots did not correspond to any of the following substances: adrenaline, 3,4-dihydroxyphenylserine, isoprenaline, epinine, or homovanillic acid. In general these nonidentified metabolites (peaks IA, IIA, IB, VB, VIB, IC, VC) never accounted for more than 4% of the total radioactivity.

Influence of Pyridoxal Phosphate

When the basic incubation medium was supplemented with amounts of pyridoxal phosphate equivalent in concentration to the substrate, the radioactivity of peak I increased from 1% to a value of 41% of the original activity (Table II). This peak had identical chromatographic properties with the DOPA – pyridoxal phosphate Schiff base complex prepared by the method of Schott and Clark (11). Therefore it is probable that in the incubation mixture this complex was formed nonenzymatically. Since only 28% of the

original DOPA (peak III) was found after incubations with pyridoxal phosphate as compared with 59% for the control, it appears that both D- and L-DOPA contribute to Schiff base formation.

TABLE II

INFLUENCE OF PYRIDOXAL PHOSPHATE ON THE DISTRIBUTION OF RADIOACTIVITY AMONGST THE METABOLITES OF DL-DOPA-α-C^{14}

	Relative distribution in %	
Peak No.	Control	Pyridoxal phosphate (117 μM.)
1	1.0	41.0
2	4.5	4.0
3	59.0	28.0
4	15.0	8.0
5	2.0	1.5
6	2.0	1.0
7	8.0	5.0

NOTE: Solvent system: *n*-butanol: 1 *N* HCl (4:1), 35 hours. Incubation medium: 2 ml. of 25% medullary homogenate, 0.4 ml. phosphate buffer pH 7.4, and 0.1 ml. of 0.005 *M* DL-DOPA-α-C^{14} (1 mg. in 1 ml.). Total volume: 3 ml. Conditions: incubated 2 hours at 37° C.

Discussion

The data presented in this study reveals that following incubation of radioactive DL-DOPA-α-C^{14} with bovine adrenal medulla homogenates six radioactive metabolites are present in addition to the recovered substrate. Five of these substances have been identified using the criteria of admixture chromatography.

Approximately 60% of the radioactivity is still present as DOPA. This may be taken to indicate that there was a large excess of the substrate. It is, however, more likely that the residual DOPA is largely the D-isomer, which is not decarboxylated by the DOPA decarboxylase (1). A similar finding was reported by Demis, Blaschko, and Welch (3).

The finding of large amounts of 3,4-dihydroxyphenylacetic acid indicates that dopamine is largely catabolized by amine oxidase. This enzyme is known to exist in the bovine adrenal medulla (8).

Noradrenaline in quantities comparable with those found by Demis *et al.* has been obtained in the present experiments. Dopamine thus appears to be the immediate precursor of noradrenaline. The direct conversion of dopamine to noradrenaline has recently been shown to occur in acetone-dried powder of bovine adrenal medulla (9). Theoretically, DOPA could also undergo a primary oxidation to give 3,4-dihydroxyphenylserine, which by decarboxylation has been shown to yield noradrenaline (2). The absence of 3,4-dihydroxyphenylserine in our extracts leaves in doubt the significance, if any, of this pathway for the formation of noradrenaline.

One minor radioactive spot (peaks IIA, IB, IC) which accounts for 1% of the initial radioactivity has been tentatively identified as DOPA – pyridoxal phosphate Schiff base complex. Because of the small concentration of isotope in this peak, its positive identification in every instance was not attempted.

The data affords no evidence for the conversion of noradrenaline to adrenaline by a transmethylation reaction.

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NEUTRAL FAT OF THE PERIRENAL FAT DEPOTS[1]

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Abstract

The possibility was investigated that perirenal fat depots which have high levels of neutral fat also have high levels of phospholipid and free cholesterol associated with increased ability to actively store fat. Lipid and water levels per unit nonlipid dry weight were measured upon perirenal fat depots in 27 male and 24 female albino rats. Mean levels of water, free cholesterol, and phospholipid were significantly higher in female than in male rats. In perirenal depots containing high levels of neutral fat, (*a*) weights of the organ were low in females ($P = 0.01$ to 0.02) but not significantly so in males ($P = 0.1$ to 0.4), (*b*) levels of phospholipid were unaffected, and (*c*) levels of ester cholesterol, free cholesterol, and water were high in both males and females ($P < 0.001$ to 0.05). The results indicate that storage of increased levels of neutral fat in perirenal fat depots of the albino rat is associated with increased levels of cholesterol, cholesterol esters, and water per unit nonlipid dry weight.

Introduction

There is a considerable body of evidence in favor of the concept that fat storage is an active, rather than a passive, function of adipose tissue (1, 2, 11, 14, 15, 16, 17, 18, 19, 20). Bloor (3) has noted that phospholipid and cholesterol levels of certain tissues may increase with increase in the particular physiological function of the tissue in question. Conceivably, therefore, the phospholipid and cholesterol levels of adipose tissue might be higher, the higher the level of neutral fat. This possibility was investigated in the perirenal fat depots of albino rats.

Method

The perirenal fat depots (12, 13) were dissected and weighed in 51 healthy, adult, albino rats. The animals were originally a Wistar strain which has been inbred in the animal quarters of the Department of Pharmacology at Queen's University since 1937. They were fed Purina Fox Chow Checkers and water ad libitum. Data concerning age, body weight, and weight of the perirenal fat depots are summarized in Table I. Aliquots of perirenal fat were weighed for lipid and water analysis by methods which have been previously noted (8), with the following exceptions. The large amount of neutral fat in these extracts was found to contaminate cholesterol digitonide and phospholipid during their precipitation and separation from other lipids. The number of washings, with suitable solvents, necessary to remove contaminant neutral fat was determined upon extracts containing known amounts of the several lipids in a range of concentrations covering the range found in perirenal fat depots. It was found that two washings of 10 ml. of each of redistilled acetone and diethyl ether removed contaminant neutral fat from

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digitonin-precipitated cholesterol by the oxidative technique of Boyd (4). Similarly, one washing with 9 ml. of dried and redistilled acetone removed contaminant neutral fat (in up to 1000 parts to one part of phospholipid) from phospholipid which had been precipitated by acetone and magnesium chloride (4). The levels of water and lipids were calculated as g. per 100 g. nonlipid dry weight of perirenal fat depots.

Results and Discussion

A summary of the data upon all males and all females as groups is presented in Table I. It may be noted that while most of the lipid is neutral fat, there are appreciable levels of phospholipid and especially of cholesterol and cholesterol esters. This is not apparent when levels are calculated per unit wet weight or dry weight. The levels of free cholesterol and phospholipid are of the order of those found in skeletal muscle (6), diaphragm (7), trachea (7), and skin (8) of rats. The levels of cholesterol esters are higher than those found in most rat tissues and comparable to those found in a rat tumor (7). The water level is below that of most rat tissues.

There were several differences due to sex of the albino rat. Females had significantly higher levels of water, phospholipid, and free cholesterol in their perirenal fat depots (Table I). The mean value for neutral fat was higher in females, but with a *P* value of 0.1 of being the same as the mean of the males.

Scatterplots were made with levels of neutral fat (in g. per 100 g. nonlipid dry weight) on the ordinate or *Y* axis and one of the other measurements noted in Table I on the abscissa or *X* axis. Estimating equations were solved by the method of least squares, using the two normal equations of

TABLE I

MEASUREMENTS UPON THE PERIRENAL FAT DEPOTS OF ALBINO RATS

Measurement	Males, $N = 27$ (mean ± standard error)	Females, $N = 24$ (mean ± standard error)	P (males equal females)
Age of rats, days	295 ± 15	314 ± 23	0.5
Body wt. of rats, g.	389 ± 10	267 ± 5.5	< 0.001
Wet wt. of depots, g.	3.28 ± 0.36	1.75 ± 0.21	0.001
Wet wt. of depots, % body wt.	0.81 ± 0.09	0.64 ± 0.06	0.1
Nonlipid dry wt. of depots, % body wt.	0.30 ± 0.05	0.20 ± 0.04	0.2
Total lipid*	246 ± 56	415 ± 84	0.1
Neutral fat*	243 ± 56	410 ± 83	0.1
Total fatty acids*	232 ± 51	391 ± 80	0.1
Total cholesterol*	0.902 ± 0.239	1.364 ± 0.411	0.3
Ester cholesterol*	0.566 ± 0.228	0.629 ± 0.386	0.3
Free cholesterol*	0.336 ± 0.060	0.735 ± 0.113	0.005
Phospholipid*	1.31 ± 0.22	3.16 ± 0.73	0.02
Water*	51 ± 9.6	90 ± 18	0.05

*Expressed as g. per 100 g. nonlipid dry weight of perirenal fat depots.

TABLE II

A CORRELATION OF LEVELS OF NEUTRAL FAT (Y AXIS) WITH WEIGHT AND LEVELS OF CHOLESTEROL, PHOSPHOLIPID, AND WATER (X AXIS) IN THE PERIRENAL FAT DEPOTS

Abscissal measurement (X)	Estimating equation for ordinate (Ye)†	Coefficient of correlation (r)	P (r equals zero)
	Males, $N = 27$		
Wet wt.*	$344 - 122\ X$	-0.182	0.4
Nonlipid dry wt.*	$323 - 350\ X$	-0.311	0.1
Phospholipid†	$241 - 0.88\ X$	$+0.003$	1.0
Ester cholesterol†	$190 + 92.9\ X$	$+0.364$	0.05
Free cholesterol†	$-47 + 866\ X$	$+0.882$	< 0.001
Water†	$-72 + 6.22\ X$	$+0.944$	< 0.001
	Females, $N = 24$		
Wet wt.*	$767 - 558\ X$	-0.458	0.02
Nonlipid dry wt.*	$607 - 1005\ X$	-0.510	0.01
Phospholipid†	$307 + 34.5\ X$	$+0.175$	0.4
Ester cholesterol†	$328 + 128\ X$	$+0.593$	0.001
Free cholesterol†	$94 + 428\ X$	$+0.575$	0.005
Water†	$31 + 4.2\ X$	$+0.892$	< 0.001

*Expressed as per cent of body weight.
†Expressed as g. per 100 g. nonlipid dry weight of perirenal fat depots.

Croxton (10). The coefficient of correlation or r was determined by Pearson's product-moment formula and a t test applied to the probability of r being zero (10). The results are listed in Table II.

The higher the level of neutral fat, the lower was the wet weight and nonlipid dry weight of perirenal fat depots in the female rats. These correlations were in the same direction in male rats but the probabilities of their being zero were 0.4 and 0.1 respectively. It will be noted that wet weight and nonlipid dry weight are entered in Table II as per cent of body weight. When calculated as g. absolute weight, correlation with neutral fat yielded coefficients (r) similar to those given in Table II. These results recall to mind the histological observation that when fat cells are loaded with fat, there is very little else present (18).

Reference to Table II will indicate that no correlation was found between the level of phospholipid and the level of neutral fat in perirenal fat depots. No evidence was found in favor of the possibility, noted in the introductory paragraph above, that phospholipid levels might be higher the higher the levels of neutral fat.

In both males and females, the levels of ester cholesterol, free cholesterol, and water were higher in perirenal depots which contained higher levels of neutral fat. In 11 animals, the levels of free cholesterol were over 1 g. per 100 g. nonlipid dry weight and in the range of free cholesterol levels found in organs such as liver (8), testicle (5), lung (8), thymus gland (7), and kidney

(9). While water levels were elevated in perirenal depots containing high levels of neutral fat, the highest levels of water reached did not approach those found in most tissues except possibly skin (8). Of particular interest, as noted above, were the high levels of cholesterol esters.

These results indicate, therefore, that the perirenal fat depots in the albino rat which accumulate the more neutral fat, relative to nonlipid dry weight, have (*a*) less nonlipid dry weight in females, possibly in males, (*b*) higher levels of ester cholesterol, free cholesterol, and water, and (*c*) no change in levels of phospholipid, per unit nonlipid dry weight.

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PUNGENIN: A GLUCOSIDE FOUND IN LEAVES OF PICEA PUNGENS (COLORADO SPRUCE)[1]

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Abstract

A new crystalline glucoside named pungenin (m.p. 198–199° C., $[\alpha]_D^{22}$ = −96.8°, water) was isolated from leaves or leafy twigs of *Picea pungens* Engelm. collected during the winter months. It comprised about 5% of the dry weight. Hydrolysis of this glucoside gave 3,4-dihydroxyacetophenone and D-glucose. Methylation of pungenin gave a crystalline pentamethyl ether (m.p. 97° C.) which gave isoacetovanillone on hydrolysis, and was found to be identical with the methyl ether obtained by complete methylation of isoacetovanillone-β-D-glucopyranoside. Pungenin is thus 3-(β-D-glucopyranosyloxy)-4-hydroxyacetophenone, a monoglucoside of 3,4-dihydroxyacetophenone.

An investigation of the water-soluble constituents of leaves of *Picea pungens* Engelm. (Colorado spruce) has resulted in isolation of a new crystalline compound in high yields (5% of the dry weight). Examination of this compound showed it to be a glucoside which gave, on acid hydrolysis, 3,4-dihydroxyacetophenone and D-glucose in approximately equimolar amounts. The analytical data agreed best with a monoglucoside of 3,4-dihydroxyacetophenone. Since no mention of such a glucoside could be found in the literature the compound was named "pungenin", following the usual custom of naming naturally occurring glucosides after the plant in which they are first found.

It was necessary to find which phenolic hydroxyl was involved in the glucosidic linkage. Methylation of pungenin, followed by hydrolysis, gave isoacetovanillone (3-hydroxy-4-methoxyacetophenone) and 2,3,4,6-O-tetramethyl-D-glucose. This shows pungenin to be 3-glucopyranosyloxy-4-hydroxyacetophenone.

Further proof of structure was obtained by synthesis. Isoacetovanillone-β-D-glucopyranoside was prepared from isoacetovanillone (sodium salt) and acetobromoglucose. Methylation of this glucoside gave a methyl ether identical with that obtained by methylation of pungenin. This synthesis confirms the structure based on hydrolysis of methylated pungenin and further shows it to be a β-glucoside. Pungenin is thus 3-(β-D-glucopyranosyloxy)-4-hydroxyacetophenone.

Naturally occurring glucosides derived from acetophenone are well known. The glucoside of *p*-hydroxyacetophenone (picein) has been found in leaves of Norway spruce (*Picea excelsa* Link.) (17) and in *Salix discolor* Muhl. (6). The structure of this glucoside was proved by hydrolysis (3, 17) and by synthesis (7). Acetovanillone-β-glucoside (androsin), first isolated from the rhizomes of dogbane (*Apocynum androsaemifolium* L.) (12), has also been

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synthesized (8, 9). The tree peony (*Paeonia arborea* Donn.) contains a glucoside of 2-hydroxy-4-methoxyacetophenone (10, 11, 13). However, no previous mention of any glucoside with the same structure as pungenin could be found.

Experimental

Isolation of Pungenin

Twigs were collected from the ends of branches of *Picea pungens* on March 15. These twigs had about 45% dry matter, 80% of which was contributed by the leaves. Leaves formed during the previous two summers were present. The isolation of the glucoside from leafy twigs is described here; other experiments on leaves separated from the stems have given about the same yield of pungenin as have also twigs collected in January and February.

The twigs (total fresh weight, 176 g.) were cut into sections about 1 cm. long and disintegrated in 3 liters of hot 80% ethanol in a heavy duty (1 gal. capacity) Waring blendor. Good pulping was obtained in about 3 minutes. The hot mixture was filtered and the pulp washed with about 200 ml. of hot 80% ethanol. The filtrate was concentrated to about 600 ml. under reduced pressure and extracted three times with ethyl ether to remove lipids and plastid pigments. The aqueous phase was concentrated to about 400 ml. and deionized by a column containing 100 ml. of Amberlite IR-120-H resin above 100 ml. of Amberlite IR-4B resin. The deionized solution was evaporated to give about 21.8 g. of a thick sirup. This was redissolved in about 50 ml. of water and chromatographed on a column (3.8 × 40 cm.) made by mixing equal volumes of Darco-G-60 charcoal and Celite 545. The sample was washed in with water and the column was developed by gradient elution (2) using a linear gradient between water and 60% ethanol (about 1200–1500 ml. of each). After 24 hours the ethanol concentration of the effluent had reached about 20% and the sugars (glucose, sucrose, and raffinose) had been eluted (20). The column was then washed with about 200 ml. of absolute ethanol and, using a linear gradient between ethanol and benzene, fractions of about 80 ml. were collected. They were evaporated under an air stream on a steam bath until nearly dry and then dried at room temperature. The pungenin was obtained in fractions 10 to 17, counting from the time the column was washed with ethanol. These fractions were combined, using 100 ml. of hot ethanol, and the solution, which already contained some suspended solid, was treated with an equal volume of petroleum ether and allowed to stand at 3° C. overnight. This deposited 3.02 g. of the crude glucoside. It was dissolved in hot ethanol, an equal volume of petroleum ether was added, and the mixture cooled overnight. This gave 2.40 g. of glucoside, m.p. 193–195° C.

The glucoside is quite soluble in hot water. Three recrystallizations from water raised the melting point to 198–199° C.; further recrystallization from water did not raise the melting point beyond this. The purified sample

showed $[\alpha]_D^{26} = -96.8°$ ($c = 0.53$, water). An aqueous solution gave a bluish-gray color with ferric chloride. Microanalysis gave C = 53.9%, H = 5.52%. Calculated for $C_{14}H_{18}O_8$: C = 53.5%, H = 5.73%. Analyses for methoxyl and Kjeldahl nitrogen were negative.

Hydrolysis of Pungenin and Identification of the Products

One gram of pungenin (m.p. 195° C.) was refluxed 90 minutes with 50 ml. of normal sulphuric acid. The acid solution was cooled and filtered through a 2.5 × 15.5 cm. charcoal–Celite column. The column was washed with water until the effluent was free of acid (130 ml.) and then with another 50 ml. of water. It was then washed with 60 ml. of 50% ethanol followed by 50 ml. of ethanol and finally by 130 ml. of benzene–ethanol (50/50, v/v).

The first fraction, containing the sulphuric acid, was neutralized by $BaCO_3$ and filtered. The filtrate and the other fractions were evaporated under a stream of air at room temperature. The aqueous fractions gave 681 mg. of a sirupy residue. The fraction eluted by 50% ethanol contained only 29 mg. of material while the ethanol and ethanol–benzene fractions gave 453 mg. of a pale-yellow crystalline material, m.p. 117° C.

The sirupy residue was found to contain a component with the same mobility as glucose by paper chromatography using *n*-butanol/acetic acid/water (4 : 1 : 1.8) and, as well, *p*-anisidine hydrochloride spray reagent. The sirup crystallized on trituration with ethanol to give 336 mg. of crude glucose. This was recrystallized once from a mixture of water, methanol, and ethanol to give 199 mg. of D-glucose, $[\alpha]_D^{24} = 52.4°$, at equilibrium ($c = 2$, water). A portion (100 mg.) was acetylated with acetic anhydride (0.6 ml.) and anhydrous sodium acetate (50 mg.) at 100° C. for 1 hour. This gave 184 mg. of the crude pentaacetate which was recrystallized from 95% ethanol to give 77 mg., m.p. 132–133° C. A mixed melting point with an authentic sample of 1,2,3,4,6-pentaacetyl-β-D-glucose showed no depression.

The crystalline aglycone (m.p. 117° C.) was dissolved in hot water, treated with a little charcoal, and filtered. Four-fifths of this filtrate was evaporated under an air stream to give 293 mg. of crystals (m.p. 119° C.). The remaining one-fifth of the solution was treated with 25 ml. of 0.5% 2,4-dinitrophenylhydrazine (in 2 *N* hydrochloric acid) at 100° C. for a few minutes. A red hydrazone precipitated; it was filtered out, and washed with water, and dried at 105° C. to constant weight—yield 174 mg. This hydrazone was recrystallized from methyl cellosolve (once) and from ethanol (twice) to give red crystals, m.p. 280° C. (decomp.). Microanalysis showed C = 50.9%, H = 3.66%, Dumas N = 16.91%. Calculated for $C_{14}H_{12}O_6N_4$: C = 50.6%, H = 3.64%, N = 16.86%. This formula corresponds to a 2,4-dinitrophenylhydrazone of a ketone with the empirical formula $C_8H_8O_3$.

The crystalline aglycone (m.p. 119° C.) was recrystallized from 2–3 ml. of water to give crystals, m.p. 120–121° C. These were recrystallized from a mixture of benzene and ethyl acetate to give prismatic needles, m.p. 122° C. Microanalysis gave C = 63.0%, H = 5.30%, Rast mol. wt. = 142. Calculated for $C_8H_8O_3$: C = 63.1%, H = 5.26%, mol. wt. = 152. This

formula agrees with that predicted from analysis of the 2,4-dinitrophenylhydrazone and suggests a dihydroxyacetophenone. The melting point of the ketone (122° C.) did not agree closely with that of any of the known dihydroxyacetophenones. The acetate was prepared from 130 mg. of crystalline aglycone, using acetic anhydride in pyridine; 148 mg. of crystals, m.p. 87° C., were obtained after recrystallization from dilute ethanol. This melting point agrees with the figure given by Voswinckel (18) for 3,4-diacetoxyacetophenone so it was considered possible that the aglycone was 3,4-dihydroxyacetophenone although its melting point has been given as 116° C. by several investigators (4, 14).

An authentic sample of 3,4-dihydroxyacetophenone was prepared from catechol diacetate by the method of Rosenmund and Lohfert (14). It was purified by crystallization from water and found to have the same melting point (122° C.) as the compound obtained by hydrolysis of pungenin. A mixed melting point determination and comparison of the X-ray powder diffraction diagrams and infrared absorption spectra showed the two materials to be identical, thus identifying pungenin as a glucoside of 3,4-dihydroxyacetophenone. It may be noted in passing that the synthetic method of Rosenmund and Lohfert, applied to guaiacol acetate, did not give 3,4-dihydroxyacetophenone as they reported (14) but acetovanillone instead (48% yield).

Pentamethyl Pungenin

Pungenin (0.80 g.), m.p. 196–197° C., was dissolved in a mixture of water (8 ml.) and methanol (3 ml.). The mixture was stirred by a Teflon-coated magnetic stirrer at 25° C. A slow stream of nitrogen was bubbled through the apparatus during the methylation. Redistilled dimethylsulphate and sodium hydroxide (100 g. NaOH plus 175 ml. of water) were used following the schedule recommended by Evans *et al.* (5) for methylation of methyl glucoside. Dimethylsulphate (2 ml.) was added all at once and then 4 ml. of alkali was added dropwise during 90 minutes. Next, 8 ml. of alkali and 4 ml. of dimethylsulphate were added at equivalent rates during 2 hours. The temperature was then raised gradually to 60° C. and more dimethylsulphate (2 ml.) added during the next 2 hours. The temperature was held at 60° C. for 4 hours and then at 45° C. overnight. The mixture was neutralized (pH 5–6) with dilute sulphuric acid, diluted to 150 ml. with water, and extracted for 5 hours with chloroform in a continuous extractor. The chloroform extract was dried by anhydrous sodium sulphate and evaporated to give 1.015 g. of a gum containing 25.6% methoxyl. This gum was remethylated as above, methanol being added whenever a gum separated from the mixture. The product was extracted from the strongly alkaline mixture with chloroform (3 hours' extraction) to give 841 mg. of a gum which solidified on standing. It was washed with petroleum ether to give 775 mg., m.p. 43–53° C. This was recrystallized from aqueous ethanol to give 241 mg., m.p. 93–95° C. Another recrystallization from water containing a

little ethanol raised the melting point to 96° C. Two more crystallizations from methanol–water gave a purified sample for analysis, m.p. 97° C., $[\alpha]_D^{22} = -83.9°$ ($c = 0.5$, ethanol). Microanalysis gave: C = 59.4%, H = 7.34%, OCH_3 = 40.3%. Calculated for a pentamethyl ether $C_{19}H_{28}O_8$: C = 59.36%, H = 7.34%, OCH_3 = 40.36%. The low melting fractions were remethylated once more and the products worked up to raise the total yield of this pentamethyl ether (m.p. 96° C.) to 430 mg. (44%).

Hydrolysis of Pentamethyl Pungenin

Pentamethyl pungenin (377 mg.) was refluxed 3 hours with 30 ml. of *N* sulphuric acid. The hydrolyzate was cooled and passed through a 1.9 × 20 cm. charcoal–Celite column. Gradient elution with water containing increasing amounts of ethanol gave, on evaporation, 202 mg. of a crystalline substance, m.p. 70–72° C. The column was then washed with ethanol, and elution continued with 50% ethanol–benzene (v/v). The alcohol–benzene fractions on evaporation gave 144 mg. of a colorless crystalline substance, m.p. 88–89° C. This was recrystallized from water to give 120 mg., m.p. 91° C. This compound was proved to be isoacetovanillone by a mixed melting point with an authentic sample (15, 16) and by comparison of the X-ray diffraction patterns.

The substance (m.p. 70–72° C.) obtained from the alcohol–water eluates was recrystallized twice from petrol containing a little ethyl ether but the melting point was not raised above 73° C. This is lower than is usually found for 2,3,4,6-O-tetramethyl-D-glucose (19). It was possible that changes had occurred on the charcoal column which made purification difficult. Another sample (100 mg.) of pentamethyl pungenin was hydrolyzed as above, the hydrolyzate passed through a column containing 7–8 ml. of IR-4B resin, and the neutral effluent evaporated to dryness under an air stream. The residue (73 mg.) was dissolved in 2 ml. of water containing 1% acetic acid and put on a column of Whatman cellulose powder (1.6 × 30 cm.) in equilibrium with the solvent. Elution was carried out with 1% acetic acid in water, 2 g. fractions being collected. Fractions 21–24 were combined and evaporated to give a residue which was extracted with hexane containing a little ethyl ether. Evaporation of this extract gave 55 mg. of colorless crystalline material (m.p. 66–68° C.). Two recrystallizations from hexane containing a little ether gave colorless needles, m.p. 89–90° C. This substance was found to be identical with 2,3,4,6-O-tetramethyl-D-glucose by a mixed melting point with an authentic sample (19) and by comparison of the X-ray diffraction patterns. Fractions 25–31 on evaporation gave 11 mg. of isoacetovanillone. Apparently most of the phenol was absorbed on the IR-4B resin since control runs showed it was readily recovered from the cellulose column.

Isoacetovanillone-β-D-glucopyranoside Tetraacetate

This was prepared by the same procedure used by Mauthner (8, 9) for synthesis of the isomer from acetovanillone. Isoacetovanillone (15, 16) (4.95 g.) was dissolved in 29.3 ml. of *N* sodium hydroxide at room temperature,

and a solution of 12.1 g. of 1-bromo-2,3,4,6-tetraacetyl-α-D-glucose (1) in 13 ml. of acetone was added during 5 minutes, with stirring, the temperature being maintained below 18° C. After 30 minutes, 13 ml. of acetone and 13 ml. of water were added. After 2 hours more, when a considerable amount of crystalline product had separated, stirring was discontinued. The volume of the mixture was decreased by about 20 ml. by evaporation under an air stream. The crystalline product was then filtered with suction, washed with water, and recrystallized from methanol while still moist. The crystals were washed with petrol (b.p. 30–60° C.) and dried under reduced pressure at 60° C. This gave 6.42 g. (43.5%), m.p. 157° C., $[\alpha]_D^{20} = -28.9°$ ($c = 0.55$, ethyl acetate). A portion recrystallized twice more from methanol had a melting point of 159° C. Found: C = 55.8%, H = 5.69%, methoxyl = 6.20%, and O-acetyl 34.7%. Calculated for $C_{23}H_{28}O_{12}$: C = 55.64%, H = 5.69%, methoxyl 6.24%, and O-acetyl = 34.65%.

Isoacetovanillone-β-D-glucopyranoside

The tetraacetyl glucoside (3.8 g.) was shaken 20 hours at room temperature with 200 ml. of 0.095 *M* barium hydroxide. The solution was neutralized to pH 6 with carbon dioxide, heated to boiling, and filtered. The filtrate was cooled under an air stream; crystallization started in about an hour. The mixture was cooled 3 hours at 3° C. and the product filtered with suction, washed with cold water, and dried at 65° C., under reduced pressure. This gave 1.89 g. (75%) with melting characteristics as described below and having a final melting point of 201–203° C. This material was recrystallized from water to give 1.72 g. having a final melting point of 202–203° C. but showing three distinct melting points when examined in the Fisher–Johns melting point apparatus. As the sample was heated it melted sharply and completely at 158° but solidified again when the temperature reached 162°. At 177° melting occurred again but was not complete; the sample gradually solidified as the temperature was raised further and then melted completely at 202–203° C. There was no further solidification when heating was continued to 226° C. The melting points of this sample were reproducible within one degree and were not changed by recrystallization. ($[\alpha]_D^{22} = -84.1$, $c = 0.5$, water.) Found: C = 54.8%, H = 6.14%, methoxyl = 9.38%. Calculated for $C_{15}H_{20}O_8$: C = 54.87%, H = 6.14%, methoxyl = 9.44%.

Isoacetovanillone-β-D-glucopyranoside Tetramethyl Ether (Pentamethyl Pungenin)

Isoacetovanillone-β-glucoside (1.0 g.) was methylated with dimethyl sulphate and sodium hydroxide, using the procedure given above for the methylation of pungenin. This gave 0.78 g. of material extracted by chloroform. Crystallization from water containing a small amount of ethanol gave 0.22 g. of white crystalline material, m.p. 93° C. Two recrystallizations raised the melting point to 96° C. A mixed melting point with the pentamethyl ether obtained by methylation of pungenin showed no depression, and comparison of the X-ray diffraction patterns showed the two compounds to be identical.

Acknowledgments

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EFFECT OF COLD ENVIRONMENT AND ASCORBIC ACID ON THE RESPIRATORY QUOTIENT OF THE MONKEY (MACACUS RHESUS)[1]

Louis-Paul Dugal[2] and Guy Fortier[3]

Abstract

Long exposure to mild cold or short exposure to intense cold lowers significantly the R.Q. of monkeys. Pre-exposure for 6 months to mild cold does not seem to affect the drop in R.Q. due to intense cold. Overdosage with ascorbic acid, in all conditions used, does not modify the R.Q.

Introduction

In previous experiments (1), it was found that ascorbic acid, in large doses, prevented a fall in body and muscle temperatures, and greatly reduced the incidence of frostbite in monkeys (*Macacus rhesus*) exposed to an intense cold of $-20°$ C. during 2 hours (2). This was especially true for monkeys which had been pre-exposed to a mild cold environment (10° C.) for about 6 months.

The purpose of the present investigation was to find out if, using exactly the same experimental conditions as the ones described in previous papers, a cold environment lowers the R.Q. of monkeys as it does the R.Q. of small mammals like hamsters (5), rats (4, 6), and guinea pigs (3); in the case that such an effect would be found in monkeys, it was also planned to see if the lowering of the R.Q. could be modified by varying the intake of ascorbic acid.

Methods

Samples of air were obtained from the animals fasted for 20 hours, kept at rest, immobilized in wooden boxes already described (1), at an environmental temperature of 22° C. (all animals were kept at that temperature during the collection of air, whether they were exposed to cold or not), and breathing *for 2 hours* into a mask equipped with one-way valves. *Only the air of the last half hour* was collected in a Douglas bag and analyzed for CO_2 and O_2 with a Beckman oxygen analyzer.

The protocol of the experiments appears in Table I. From that table, it is seen that 16 monkeys were used at room temperature, seven treated with 25 mg. per day of ascorbic acid, nine with 325 mg. per day. On the animals of the first group at room temperature 73 determinations of R.Q. were made, and 106 on those of the second group at the same temperature. Of the seven monkeys of the first group, three, after R.Q. determinations at room temperature were completed, were placed in a mild cold environment (10° C.) for 6 months and then subjected again to R.Q. determinations; finally, those

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TABLE I

Temperature	Ascorbic acid per day: 25 mg.	Ascorbic acid per day: 325 mg.
Room	3*(34) + 2(22) + 2(17) = 7(73)	3(33) + 2(21) + 4(52) = 9(106)
Mild cold (6 months)	3(28) + 2(16) = 5(44)	3(28) + 2(16) = 5(44)
Intense cold (2 hours)	3(32) + 2(21) = 5(53)	3(29) + 4(39) = 7(68)

* = Number of monkeys.
NOTE: The number of R.Q. determinations is in parentheses.

same three monkeys were exposed frequently (about once a week) to an intense cold of $-20°$ C. for 2 hours, and the R.Q. due to that intense cold measured (32 determinations). As the table indicates, some of the monkeys were submitted only to mild cold, and some were transferred directly to intense cold exposure, without any pre-exposure to mild cold.

Results

Table II shows the effect of time on the R.Q. of monkeys kept at room temperature. Such an experiment was designed so as to eliminate the possibility that any change in R.Q., obtained when the monkeys were exposed to cold, could be attributed to the sampling method: it was indeed possible that without any change in the surrounding temperature, adaptation to the sampling procedure (mask and immobilization) could lower the R.Q. with time. This point has been clarified by comparing the results obtained in taking a series of R.Q. after training the animals for 2 months to breathe with a mask while immobilized for 2 hours (about twice a week) and another series after 6 months of the same training. As Table II shows, there is no difference in R.Q. between the two series of experiments.

Monkeys exposed to a mild cold environment (10° C.) for 6 months show a drop in R.Q. which is significant (Table III). More important and significant is the fall in R.Q. due to a short exposure to intense cold (2 hours at $-20°$ C.) (Table III).

The fall in R.Q. due to intense cold does not seem to be affected by the fact that monkeys have been pre-exposed or not to mild cold (Table IV). At least, the difference between the two series of experiments is not significant.

TABLE II

EFFECT OF TIME ON THE R.Q. OF MONKEYS KEPT AT ROOM TEMPERATURE

Months of training at room temp.	No. animals	No. determinations	Av. R.Q.
2	6	67	0.829 ± 0.011*
6	6	62	0.821 ± 0.014

*Standard error.

TABLE III

EFFECT OF EXPOSURE TO MILD COLD AND INTENSE COLD ON THE R.Q. OF MONKEYS

Temperature	No. animals	No. determinations	Av. R.Q.	Significance of difference
Room temp.	10	110	0.842 ± 0.011*	$p = 0.01$
10° C.	10 (the same as at room temp.)	85	0.795 ± 0.011	
Room temp.	12	129	0.825 ± 0.009	$p < 0.001$
−20° C.	12 (the same as at room temp.)	121	0.754 ± 0.008	

*Standard error.

TABLE IV

EFFECT OF INTENSE COLD EXPOSURE ON THE R.Q. OF MONKEYS PRE-EXPOSED AND NOT PRE-EXPOSED TO MILD COLD

Temperature	No. animals	No. determinations	Av. R.Q.	Significance of difference	
		Animals pre-exposed			
Room	6	67	0.829 ± 0.011*	$p = < 0.02$	$p = < 0.001$
10° C. (6 months)	6 (the same as at room temp.)	56	0.796 ± 0.008	$p = < 0.01$	
−20° C.	6 (the same as at room temp.)	61	0.745 ± 0.013		
		Animals not pre-exposed			
Room	6	62	0.821 ± 0.014	$p = < 0.01$	
−20° C.	6 (the same as at room temp.)	60	0.764 ± 0.006		

* = Standard error.

TABLE V

EFFECT OF DIFFERENT DOSES OF ASCORBIC ACID ON THE FALL OF R.Q.

Temperature	25 mg. per day ascorbic acid			325 mg. per day ascorbic acid		
	No. animals	No. determinations	Av. R.Q.	No. animals	No. determinations	Av. R.Q.
Room	7	73	0.830 ± 0.018*	9	99	0.837 ± 0.008
Mild cold 10° C.	5	44	0.804 ± 0.017	5	41	0.785 ± 0.012
Intense cold −20° C.	5	53	0.751 ± 0.014	7	68	0.757 ± 0.0096

* = Standard error.

The effects of different doses of ascorbic acid have been studied and the results are tabulated in Table V. Overdosage with ascorbic acid does not seem to affect the R.Q. as compared to normal dosage.

Discussion

Our results obtained on monkeys appear to confirm the ones obtained by other workers on small animals (5, 4, 6, 3) that exposure to cold results in a marked drop in R.Q. and would agree with the theory that fat is used preferentially for extra heat production in the cold.

In animals exposed to mild cold for long periods (adapted?) there is a significant fall in R.Q. as compared to the R.Q. of control animals kept at room temperature. Such a result is in complete agreement with the one obtained by Pagé and Chénier (5).

Short exposure to intense cold causes a greater drop in R.Q. than the one obtained after long exposure to mild cold; such a drop due to intense cold is the same whether the animals have been pre-exposed to cold or not.

Résumé

Nous avons observé qu'une longue exposition à un froid modéré (six mois à 10° C.) et qu'une courte exposition à un froid intense (deux heures à −20° C.) abaissent, et de façon significative, le Q.R. des singes (*Macacus rhesus*). La pré-exposition au froid modéré n'altère en rien la baisse du Q.R. causée par l'exposition au froid intense. L'acide ascorbique, à doses massives, ne modifie pas le Q.R., tout au moins dans les conditions expérimentales où nous avons opéré.

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